

An *in vitro* investigation into the glucose regulating activity of *Gnidia canoargentea* and *Osyris lanceolata*

by

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Declaration

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Abstract

Diabetes mellitus, is an endocrine disorder characterized by insulin resistance and glucose tolerance impairment with mainly two classifications (type 1 diabetes and type 2 diabetes mellitus). Type 2 diabetes mellitus has been reported to be the third leading natural cause of death in South Africa. While mainstream Euro-Western medical treatment and management of type 2 diabetes tend to be effective, there remain strong societal and cultural beliefs in using traditional medicine. It is believed that traditional medicines offer beneficial properties with regards to managing health conditions and have less side effects compared to pharmaceutical drugs. *Gnidia canoargentea* and *Osyris lanceolata* are herbal remedies used traditionally to treat and manage diabetes in North West, South Africa. The aim of this study was to evaluate the *in vitro* glucose regulating activity of the plant extracts.

Extracts were prepared using hot water, methanol dichloromethane (DCM:MeOH) and chloroform partition which involved brewing and maceration techniques, respectively. The phytochemical constituents of the plant extracts were determined using thin layer chromatography and ultra-performance liquid chromatography. Furthermore, carbohydrate hydrolyzing enzyme (α -amylase and α -glucosidase) inhibition activity was monitored using a colorimetric method. Cytotoxicity against C2C12 mouse myoblast and Ea.hy926 cell lines were determined using the sulforhodamine B staining assay. The effect of the extracts on oxidative stress (reactive oxygen species) was assessed in Ea.hy926 cells using permeable fluorogenic dichlorodihydrofluorescein diacetate assay. Glucose uptake was assessed fluorometrically in C2C12 cells using 2-[N-(7-nitrobenz-2-oxa-1,3-dioxol-4-yl) amino]-2-deoxyglucose assay.

Phytochemical analyses revealed the presence of kaempferol-3-rutinoside, rutin (flavonoid) and Pelargonidin-3-O-rutinoside and pelargonidin-3-O-glucoside (phenolics) compounds within the plant extracts. The aqueous extracts of *O. lanceolata* ($IC_{50} = 13.79 \mu\text{g/mL}$) possessed more potent scavenging activity than the DCM:MeOH extract ($IC_{50} =$

23,51 µg/mL), whereas for *G. canoargentea*, the DCM:MeOH ($IC_{50} < 3.125$ µg/mL) extract possessed more potent scavenging activity than the aqueous extract ($IC_{50} = 3.55$ µg/mL). Moreover, the chloroform partition of *O. lanceolata* exhibited a more cytotoxic reaction (~35% cell cytotoxicity) in C2C12 cells, while the combination (*O. lanceolata* & *G. canoargentea*) plant extract had concentration dependent cytotoxicity in C2C12 cells; thus, indicating potential susceptibility of muscle tissue to the herbal remedy. Only the DCM:MeOH ($IC_{50}=35.48$ µg/mL) and the chloroform ($IC_{50}=17.48$ µg/mL) extract of *G. canoargentea* displayed inhibitory activity against α -amylase.

The aqueous and organic plant extract of the selected plant species did exhibit *in vitro* oxidative stress regulation in Ea.Hy926 cells, however the activity was insignificant compared to the positive control Trolox. In the glucose uptake study the organic extract of *G. canoargentea* improved the glucose uptake by two-folds in C2C12 cells, however was not significant in comparison to the positive control insulin. The combination plant extract was unsuccessful in upregulating glucose uptake in C2C12 cells.

Based on the results, it is evident that *O. lanceolata* and *G. canoargentea* extracts play part in reducing free-radical damage by scavenging radicals which play part in the disease progression and also inhibit the carbohydrate hydrolyzing enzyme, α -amylase. Furthermore, *O. lanceolata* and *G. canoargentea* offer minimal to no glucose regulation when extracted with water, neither does the combination of the plants offer improved activity and thus the herbal remedies studied do not offer beneficial attributes to glucose regulation when evaluated with the assays employed in this study.

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List of abbreviations

2-NBDG	2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4yl) amino]-2-deoxyglucose
ABTS	2,2-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid
ANOVA	One-way analysis of variance
ANSA	3-Amino-5-nitrosalicyclic acid
ATP	Adenosine triphosphate
AKT	Protein kinase B
CAT	Catalase
C2C12	Mouse myoblast (CRL-1772)
CSIR	Council for Scientific and Industrial Research
Cu	Copper
DCF	2',7'-Dichlorodihydrofluorescein
DCFH-DA	2',7'-Dichlorodihydrofluorescein diacetate
dH ₂ O	Distilled water
DCM	Dichloromethane
DCF	Dichlorodihydrofluorescein
DNSA	3,5-Dinitrosalicyclic acid
DM	Diabetes mellitus
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl free radical
EDTA	Ethylenediaminetetra-acetic acid
FCS	Foetal calf serum

g	Gram
<i>g</i>	Gravitational force
GLUT	Glucose transporter
GPx	Glutathione peroxidase
GrB-2	Growth factor receptor bound protein
H ₂ O ₂	Hydrogen peroxide
h	Hour/s
HL	Human lymphocytes
HPLC	High performance liquid chromatography
IC ₅₀	Half maximal inhibitory concentration
IDDM	Insulin-dependent diabetes mellitus
IGT	Impaired glucose tolerance
IRS	Insulin receptor substrate
IR	Insulin resistance
K ₂ C ₄ H ₄ O ₆	Potassium tartrate
K ₂ S ₂ O ₈	Potassium sulfate
L	Liter
L/h	Liters per hour
MAP	Mitogen-activated protein
MeOH	Methanol
MS	Mass spectrometry
Min	Minute
mL	Milliliter

mm	Millimeter
mM	Millimolar
Mn	Manganese
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaHCO ₃	Sodium bicarbonate
NIDDM	Non-insulin dependent diabetes mellitus
NF _κ B	Nuclear factor kappa B
NH ₂ Cl	Chloramine
NH ₄ Cl	Ammonium chloride
nM	Nanomolar
NMR	Nucleic magnetic resonance
NO	Nitric oxide
NO ₂ ⁻	Nitrite
O	Oxygen
OH	Hydrogen oxide
·OH	Hydroxyl radical
OH ⁻	Hydroxyl ion
ONOO ⁻	Peroxynitrite
O ₂ ⁻	Superoxide
PKC	Protein kinase c
PBS	Phosphate buffer
PI-3-Kinase	Phosphatidylinositol 3 kinase

RFI	Relative fluorescence intensity
Py	Phosphorylation
ROS	Reactive oxygen species
s	Second/s
SANBI	South African National Biodiversity Institute
SEM	Standard error of the mean
SOD	Superoxide dismutase
SRB	Sulphorhodamine B
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA	Trichloroacetic acid
THP	Traditional health practitioner
TLC	Thin layer chromatography
TRIS	Trisaminomethane
UPLC	Ultra performance liquid chromatography
USD	United State Dollar
U/mL	Unit per milliliter
UV-VIS	Ultra-violet visible spectrum
v/v	Volume/volume percentage
WHO	World Health Organisation
w/v	Weight/volume percentage
Zn	Zinc

Chapter 1: Literature review

1.1. Introduction

Diabetes mellitus (DM) is a chronic endocrine disorder that occurs when the pancreas produces insufficient insulin into the blood stream or where there is minimized tissue response to insulin in the hormone pathway action.¹ When there is insufficient insulin production to regulate the blood glucose levels, hyperglycaemia develops.² Chronic hyperglycaemia is a condition that causes metabolic disturbances to the breakdown of carbohydrates, fats and proteins through enzymatic reactions to produce essential by-products that are required by the body to function.^{2,3} The development and progression of hyperglycaemia in the absence of correct medical attention, leads to health complications such as cardiovascular disease, nephropathy, neuropathy and retinopathy.³

According to the International Diabetes Federation, more than 425 million people were diagnosed with diabetes worldwide in 2017, of which 16 million cases were on the African continent,³ and 1.8 million cases were from South Africa.³ Ninety percent of diabetes cases worldwide are type 2 diabetes mellitus (T2DM) as opposed to type 1 diabetes mellitus (T1DM).³

The expanse of diabetes worldwide corroborates with the statistics reported by the South African government in the official reports, where diabetes has been considered the 3rd leading natural cause of death.⁴ The World Health Organisation (WHO) indicated that the mortality rate due to diabetes was about 1.5 million worldwide in 2012, of which 80% of the cases were from developing countries.⁵ Furthermore, it is predicted that the death rate due to DM will increase by 50% by 2020.⁵

1.2. Diabetes mellitus

Diabetes mellitus has been commonly classified into two major classes, namely insulin dependent diabetes mellitus (IDDM) or commonly known as T1DM and non-insulin dependent diabetes mellitus (NIDDM) also known as T2DM.^{1,2} Both types may result in various health related complications, including hyperlipidemia, hyperinsulinemia, hypertension and atherosclerosis.⁶ Another type of diabetes that may be a precursor for T2DM is gestational diabetes which occurs during pregnancy when the need for insulin increases.⁷ This type of condition subsides after childbirth.⁷

i) Type 1 diabetes mellitus

Type 1 diabetes mellitus, occurs as a result of pancreatic β -cells being destroyed by the immune system leading to “full-blown” diabetes, where no insulin is produced in the body.¹ Exogenous insulin is required in this instance as a treatment in order to prevent ketoacidosis and potentially death.¹ Some of the clinical symptoms associated with T1DM include excessive urine production, vision changes, fatigue, thirst, constant hunger and weight loss.^{2,3} The occurrence of T1DM is prevalent in adolescences and rare in adults.⁸ In T1DM, the β -cells are aggressively destroyed by the immune system in a short space of time.⁸ Four autoantibodies that are targeted at certain antigens need to be assessed to confirm the development of T1DM (antigens: insulin, islet cells, IA-2 molecule and glutamic acid decarboxylase).⁸ The parameters are also used to determine the progression rate to acquiring clinical T1DM.⁸

ii) Type 2 diabetes mellitus

Type 2 diabetes mellitus is a progressive condition characterized by insulin resistance and impaired glucose tolerance (IGT).⁹ It occurs when there is a build-up of endogenous insulin in the blood stream, produced by β -cells of the pancreas, during hyperplasia and hypertrophy.^{10,11} This results in increased amylin deposit build-up in the pancreas, and as a consequence, hypotrophy and hypoplasia.^{10,11} Glucagon secretion is also increased in patients with T2DM, resulting in the continual production of glucose.¹¹ This metabolic

disorder accounts for 90% of all diabetes cases that occur worldwide.^{3,12} This condition suppresses the metabolic response of muscles, liver and adipose tissue to insulin action.¹² This metabolic condition and insulin resistance leads to hyperglycaemia.¹²

Most patients with T2DM suffer from obesity, a high percentage of fat in the abdominal region, and live sedentary life styles.^{1,2,3} The symptoms of patients who present with T2DM is similar, albeit less pronounced, than those with T1DM. Most individuals who have T2DM are only diagnosed later in life when complications are already advanced.¹ Reduction in weight, increase in activity or use of pharmaceutical treatment for hyperglycaemia may improve insulin resistance, however, the use of drug therapy has been associated with long term underlying and undesirable side-effects such as hypoglycaemia or weight gain.¹

The possibility of developing T2DM increases with age, lack of physical activity and weight gain resulting in obesity. T2DM occurs frequently in individuals who have hypertension and also in women who previously presented with gestational diabetes mellitus.³ If T2DM is left untreated, the hyperglycaemia may lead to long term macrovascular and microvascular complications such as atherosclerosis nephropathy, neuropathy and retinopathy.³

Recent studies have led to advancing our understanding of DM and the classification thereof. The new classification model includes taking into account the presence or absence of autoantibodies against β -cell antigens, age and blood glucose levels.¹³ Based on the former classification, 90% of people with diabetes had T2DM. According to the revised classification, T2DM is only present in 75% - 85% of diabetics.¹³ The remaining ~10% is now termed latent autoimmune diabetes (LAD), and only occurs in adults.¹³ The LAD subgroup, although phenotypically similar to T2DM, differs from the former in that these patients contain glutamic acid decarboxylase antibodies(a marker for insulin dependent diabetes mellitus condition).¹³ A unique aspect of LAD is that at diagnosis it is similar to T2DM, but as time progresses, it becomes more like T1DM.¹³ The newly

proposed classification looks at differentiating the types of T2DM and gives insight to how the therapy for the condition can be determined (Table 1).¹³

Table 1: Newly proposed categorization of the different clusters of T2DM.¹³

Analytical character	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Body mass index	Low	Low	High	High	Average
Glutamic acid decarboxylase antibodies	Positive	Negative	-	-	-
Insulin resistance	Present and high	Present and low	Present and high	Present and low	Present and Low
Early onset	Yes	Yes	No	No	No

- = Not applicable

1.3. Physiology of type 2 diabetes mellitus

1.3.1. Carbohydrate metabolism

Carbohydrates are complex polysaccharides present in food products, which are digested in the mouth and small intestine by enzymes into disaccharides and monosaccharides.¹⁴ The enzymatic process is initiated in the mouth, where salivary α -amylase hydrolyses the α -1,4 bond of the oligosaccharides to produce smaller polysaccharides/disaccharides which consist of glucose molecules.^{15,16}

As the digested carbohydrates reach the stomach, the enzymes are inactivated due to the acidic conditions of the environment.¹⁶ When the polysaccharides/disaccharides enter the small intestine, pancreatic α -amylase is produced and initiates the breakdown of the molecules into maltose and isomaltose (Figure 1).^{14,16}

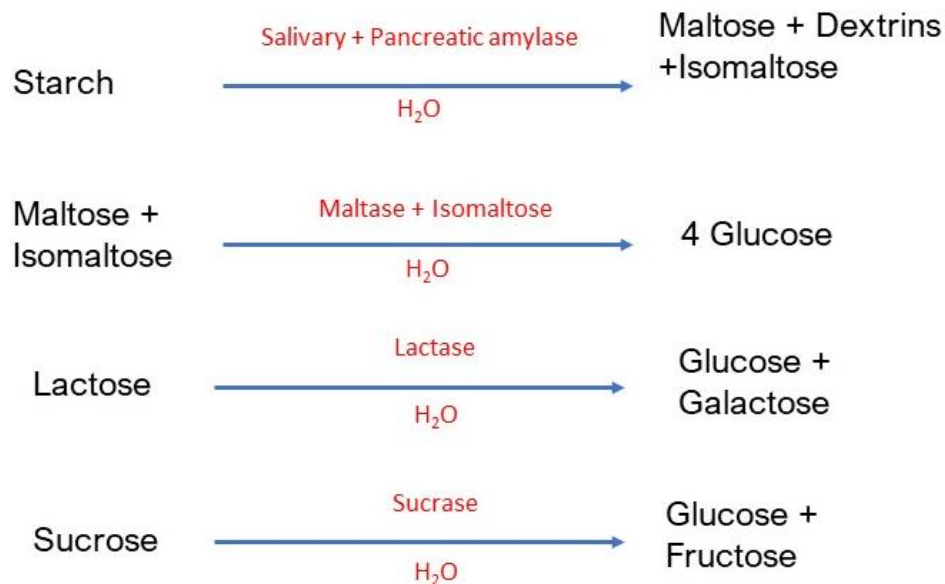


Figure 1: A diagram indicating dietary carbohydrate metabolism.¹⁷

The maltose and isomaltose molecules are then transported to the brush border in the intestine where glucose, fructose and galactose is produced (by mediation of glucosidase) which if not absorbed into skeletal and adipose tissue can leads to hyperglycaemia.¹⁶

In healthy individuals, the produced glucose is absorbed into skeletal and adipose tissue with the assistance of insulin and glucose transporter (GLUT) molecules.²⁰ Insulin is a hormone produced by the islet of Langerhans in response to increasing concentrations of glucose.^{9,20} The increased concentration of insulin results in the activation and translocation of GLUT-4 molecules from the interior of cells to the surface.¹⁹ When the GLUT molecules have populated the cell membrane, glucose uptake into muscles takes place.²⁰ The process is followed by the detachment of insulin from the cell membrane and undergoes digestion.²¹

In addition to regulating the uptake of glucose, insulin also inhibits two carbohydrate pathways that take place during the fasting state; gluconeogenesis and glycogenolysis, which are key in decreasing basal glucose concentration.²¹ Gluconeogenesis produces glucose in the liver and kidneys from amino acids and lactic acid.²⁰ Glycogenolysis converts glycogen into glucose-1-phosphate which increases the basal glucose concentration.²¹ In patients with T2DM, the insulin production is defective (produces low insulin or tissue is insensitive to internally produced insulin) and thus results in an increased concentration of glucose in the blood.¹⁹

1.3.2. Insulin and Diabetes

The beta cells from the pancreatic islet synthesis a precursor for insulin known as proinsulin.^{22,23} Proinsulin is a single chain of insulin molecules with a C-peptide.^{22,23} When the C-peptide is removed, active insulin is produced and stored in secretory granules.^{22,23} Insulin secretion is stimulated when glucose (primary stimulant) enters the beta cells through the GLUT2 transporters to produce ATP via metabolism by glycolysis.^{22,23} Other nutrients such as amino acids and fatty acid can also induce insulin secretion, while some hormones may potentiate insulin response to nutrients or inhibit secretion of insulin.^{22,23}

In a health individual, 70-100 mg/dL is the expected plasma glucose concentration after an overnight fast, which after a meal can be between 120-140 mg/dL.^{22,23} The ingestion of a meal stimulates the production of insulin.^{22,23} The insulin released by the beta cells enters the circulation and initially reaches the liver, where it binds to the heterodimeric protein receptors belonging to the family of tyrosine kinase.^{22,23} This receptor has an extracellular α -subunits attached to two transmembrane β -subunits on the cell surface.^{22,23} When insulin binds to the α -subunits, a conformational change in the β -subunits results, thus activating tyrosine kinase activity of the receptor.^{22,23} The activation and autophosphorylation of the β -subunits causes a cascade of phosphorylation of tyrosine residues on a series on intracellular proteins such as SHC-transforming protein, insulin receptor substrates, and growth factor receptor-bound protein (GrB-2), resulting in an enzymatic pathway involving phosphatidylinositol 3-kinase (PI-3-Kinase) and mitogen-activated protein kinase (MAP).^{22,23} The PI-3-Kinase pathway mediates the stimulation of glucose transport by translocation of GLUT receptors to the cell membrane (mostly GLUT 4 receptors), synthesis of glycogen and proteins and inhibit lipolysis.^{22,23} The MAP pathway also

known as Ras-Raf-MEK-ERK pathway is involved in gene expression, cell growth and differentiation (Figure 2).^{22,23}

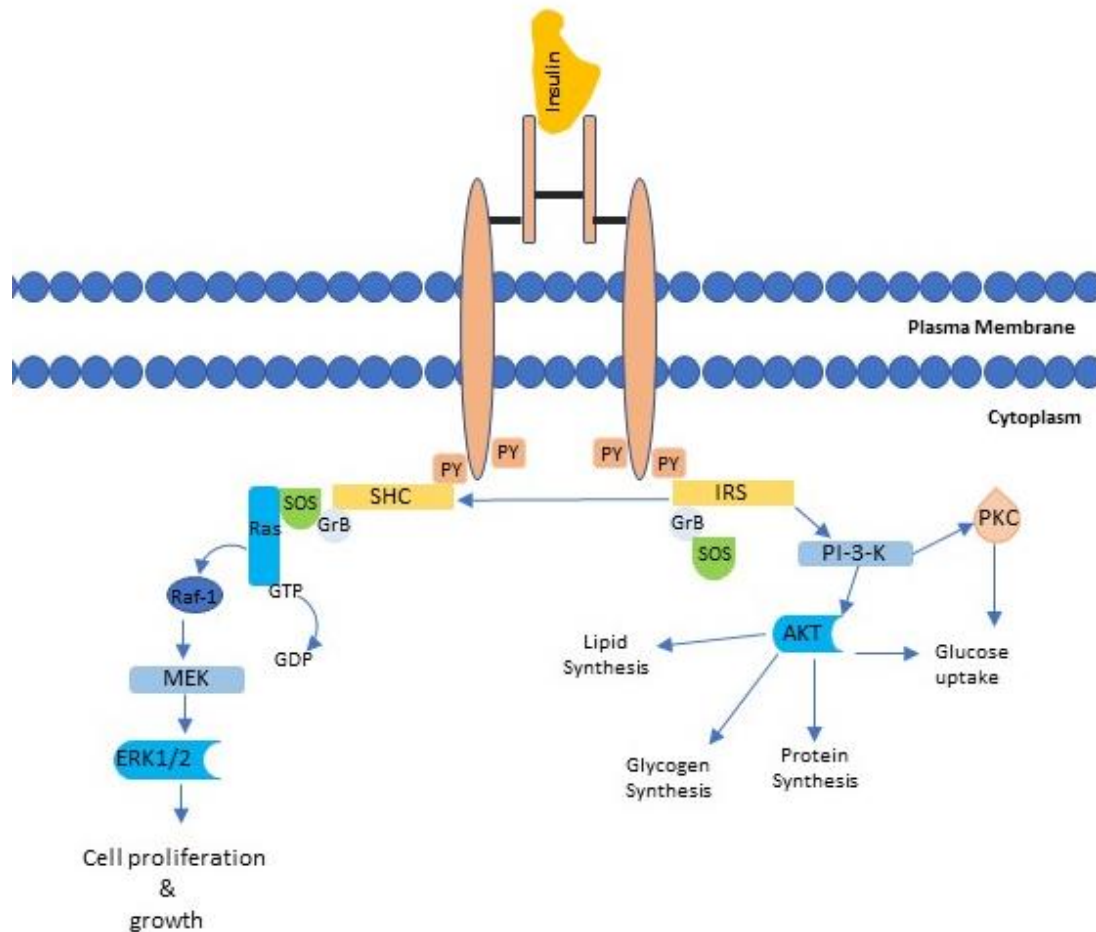


Figure 2: Intracellular insulin signaling pathway and regulation of glucose metabolism (adapted from Simonson D).²²

PY: phosphorylation, IRS: insulin receptor substrate, GrB: growth factor receptor-bound protein, PI-3-K: phosphatidylinositol 3-kinase, PKC: protein kinase C, MAPK (Ras-Raf-Mek-ERK): mitogen activated kinase, AKT: protein kinase B.

The liver is responsible for clearing ~50%-60% of the produced insulin by receptor mediated clearance before reaching the systemic circulation.^{22,23} The remaining insulin in circulation causes the removal of circulating glucose by muscles (primarily) and is stored as glycogen or is oxidized to produce ATP.²³

In individuals with insulin resistance during fasting or absorptive stage, hepatic glucose production is no longer fully restrained, thus causing a rise in fasting plasma glucose concentration.²² Glucose uptake in muscle during the postprandial state is also affected, leading to an excessive rise in plasma glucose concentration and increased hyperinsulinemia.²² This continued exposure to excess glucose leads to B-cell failure.^{22,23}

1.3.3. Oxidative stress

The production of reactive oxygen species (ROS) leads to oxidative stress, which is a central element in diabetes progression.^{24,27} In the hyperglycaemic state, glucose regulation is ineffectively controlled, and free radical production occurs.^{25,26,37} Due to the free radicals produced and acute changes of metabolism resulting from hyperglycaemia, tissue damage takes place.⁹

Reactive oxygen species are molecules and free radicals that are produced when atomic oxygen is reduced via the addition of electrons in mitochondrial electron transport during aerobic respiration.^{27,28} Under aerobic conditions, the electron transport chain in combination with cytochrome oxidase, reduces 90% of consumed oxygen into water within the mitochondrial membrane, without the production of ROS.^{25,27} In the case where there is a high concentration of glucose (hyperglycaemia), electron reflux is increased (as the cell tries to reduce the glucose level in blood) and nicotinamide adenine dinucleotide (NADH) is produced.²⁵ The enzyme conversion of glucose to polyalcohol sorbitol increases while the nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione decreases.^{25,28} Hyperglycaemia also results in increased adenosine triphosphate (ATP) to adenosine diphosphate (ADP) ratio causing the inner mitochondrial membrane potential to be hyper-polarized (partial inhibition of electron transport in complex III).²⁸ This reduces the antioxidant electives, resulting in relatively high oxidative stress that is associated with ROS production.^{29,31}

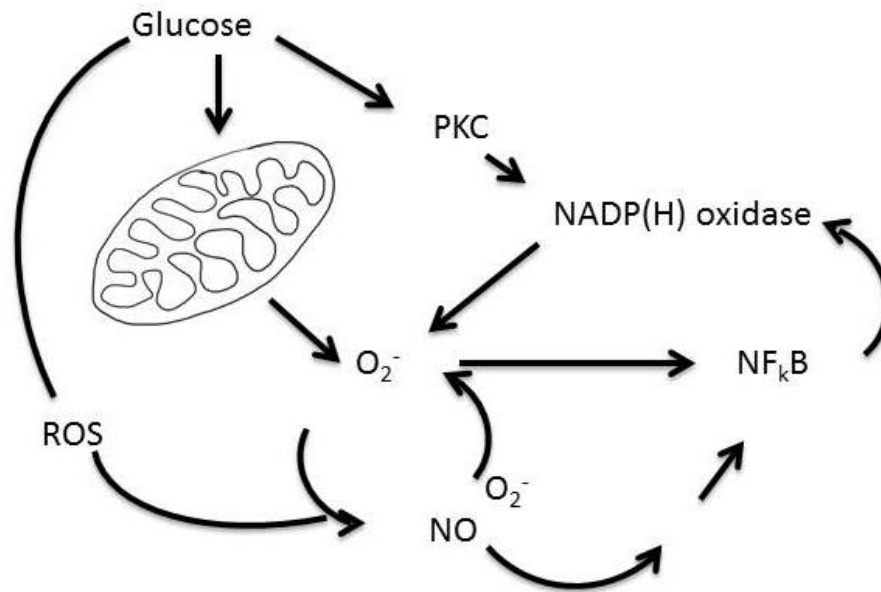


Figure 3: Oxidative stress mechanism in diabetes relating to increased glucose availability (adapted from Kim and Son).³⁰

PKC: Protein kinase C; NADP(H): Nicotinamide adenine dinucleotide phosphate; NF_κB: Nuclear factor kappa B; ROS: Reactive oxygen species; O₂⁻: Superoxide; NO: Nitric oxide

Oxidation of glyceraldehyde-3-phosphate by glyceraldehyde-3-phosphate dehydrogenase is reduced in hyperglycaemic conditions thus resulting in auto-oxidation which yields hydrogen peroxide (H₂O₂), α-ketoaldehyde and activation of protein kinase C (Figure 3).^{24,27,29} The activity of protein kinase C (PKC) is further enhanced by the presence of diacylglycerol that is elevated during hyperglycaemia.^{24,27} The activated PKC phosphorylates the cytosolic component of NADPH oxidase and activates the Na⁺-H⁺ exchanger, increasing calcium ions (Ca²⁺).³⁰ The Ca²⁺ in turn activates the NADPH and thus more ROS is generated.^{25,30} The increased oxidative stress from PKC activation also enhances endothelial nitric oxide (NO) synthase uncoupling, leading to increased free radical production.^{24,27,29}

The abnormal activation of the PKC pathway leads to a high concentration of ROS.^{27,32} The ROS species lead to insulin resistance (IR) when cellular stress sensitive pathways

are activated.³² Organs such as adipose tissue, the heart, liver and skeletal muscles develop insulin resistance.³²

The high production of ROS in hyperglycaemia leads to defective insulin expression in the β -cells, which results in insufficient levels of insulin for the insulin signal pathway.^{24,33,32} The oxidative stress in the endoplasmic reticulum has been shown to cause relatively high cell apoptosis due to the increased expression of pro-apoptotic genes in the islets.^{24,26,37} The reduced insulin levels affect adipose tissue, whereby adiponectin synthesis is reduced, while leptin, tumour necrosis factor- α (TNF- α) and IL6 are increased during insulin resistance and T2DM.^{26,32} The oxidative stress resulting from hyperglycaemia activates macrophages, which then interact with the adipocytes.²⁶ When the adipocytes interact with tumour necrosis factor- α (TNF- α) produced by macrophages, they enlarge, and produce high levels of free fatty acids (FFAs). The FFAs then bind to toll-like receptor-4 that is expressed by macrophage.^{26,32} In the absence of, or at low concentrations of adiponectin, more TNF- α is produced.²⁶ Adiponectin has the important function of enhancing glucose uptake and inhibiting hepatic glucose production.²⁶ Considering this, it is important that oxidative stress is managed in diabetic patients, by a diet rich in antioxidants.

1.3.4. Antioxidants

Within cells, the ROS molecules that are produced need to be scavenged and converted to harmless molecules.³⁴ This is done by antioxidants that form within the cell, generally in the form of enzymes.³⁴ In physiological conditions superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) act as the main antioxidant enzymes that regulate the radical species in question.³⁴ Superoxide dismutase is the first line of defense against ROS molecules in the cell.^{28,30,31,34} The SOD reacts with the superoxide and converts it into hydrogen peroxide, which requires further breakdown.^{28,230,231,34}

As hydrogen peroxide is still active and may react with other ROS molecules, the other antioxidant enzymes, catalases (in the lysosomes) and glutathione peroxidase (in the mitochondria) degrade it to water and oxygen.^{28,31} Glutathione peroxidase undergoes

regeneration after reacting with hydrogen peroxide with the assistance of NADPH (Figure 4).²³ Other non-enzymatic antioxidant mechanisms that help eliminate ROS molecules and regenerate glutathione peroxidase are vitamin A, C, E and α -lipoic acid.³⁴

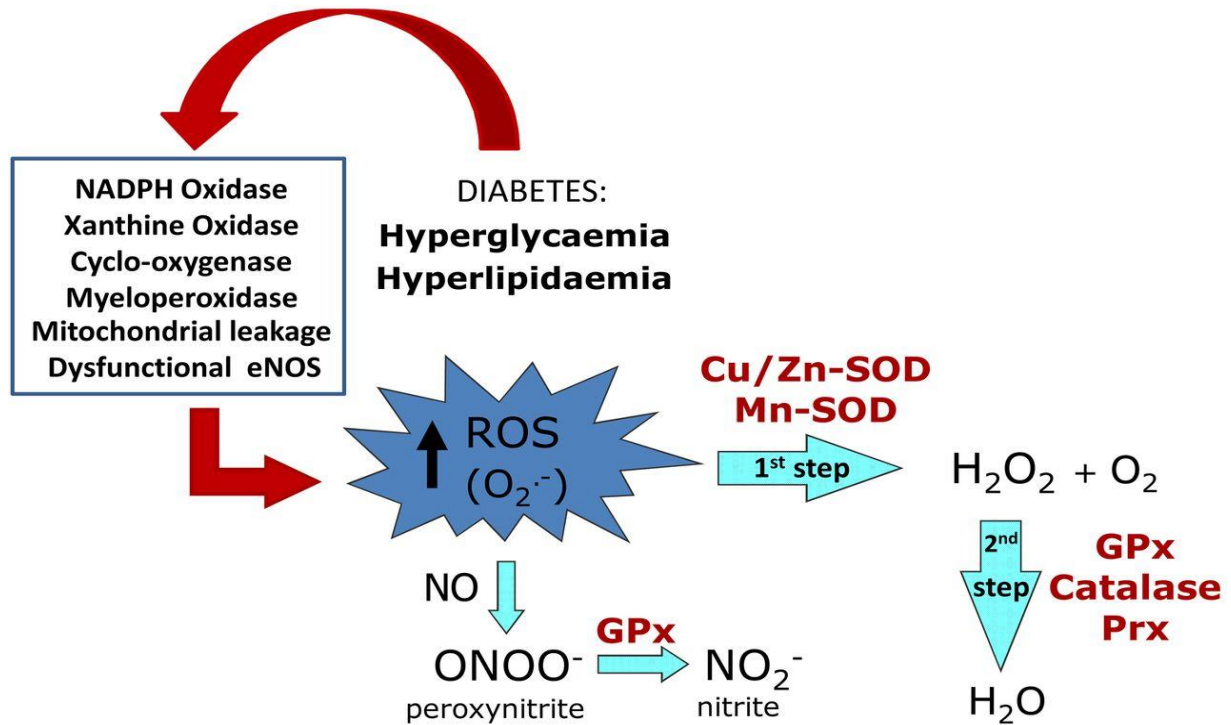


Figure 4: The enzymatic antioxidant pathway (reproduced with permission from Di Marco *et al.*)³⁴

Cu: copper; Zn: Zinc; Mn: Manganese; NO₂⁻: Nitrite; ONOO⁻: Peroxynitrite; SOD: Superoxide dismutase

During chronic hyperglycaemia, an over production of ROS occurs and the antioxidant by-products are reduced due to the reaction with the reactive species.^{23,26} The availability of enzymes in the cells are suppressed, leading to an increase in ROS production.³²

1.4. Complications resulting from diabetes

1.4.1. Nephropathy

Diabetic nephropathy is kidney damage that is an outcome of the hyperglycaemic state that results from the high blood glucose.^{32,36} Normally, blood is filtered through the kidney by entering the afferent arteriole, passing through the glomerulus and exiting through the efferent arteriole.^{32,36} During the filtrate production, the blood moves through the endothelium (lines the glomeruli capillaries), the basement membrane and the epithelium which lines the nephron.^{29,32,36} The porous endothelium with the assistance of cell's podocytes, prevent cells and negatively charged molecules and proteins from entering the filtrate produced.^{36,37} With diabetes, due to the excess glucose in blood, when the blood passes through the kidneys, glycosuria results as the glucose passes through the endothelium. Some of the excess glucose also binds to proteins, which leads to non-enzymatic glycation.^{36,37} During this process the efferent arteriole membrane is glycated causing the tissue to harden (hyaline arteriosclerosis).^{29,37} Due to this obstruction, hyperfiltration occurs, where there is increased pressure within the glomerulus and thus the glomerulus filtration rate is increased.^{29,37} The mesangial cells located in the glomerulus produce excessive structural matrix and formation of Kimmelstiel-Wilson nodules in response to the hyperfiltration, thus expanding the size of the glomerulus.^{29,36,37} The expansion of the glomerulus alters the filtration slits of the podocytes and thus creates an ineffective filtration system.^{29,36,37}

Diabetic nephropathy is a progressive complication that does not have overt symptoms, thus the only way to manage this, is by screening urine on a regular basis.^{36,37} The early stage of the condition is characterized by the excretion of 30-300 mg of albumin a day known as microalbuminuria.²⁹ Macroalbuminuria, where more than 300 mg of albumin is excreted a day, is a late stage of the condition and most likely will be followed by renal failure.²⁹

1.4.2. Neuropathy

Diabetic neuropathy is a painful condition where peripheral nerves are damaged leading to pain or numbness (the arms, legs, feet and toes are the most affected areas) due to diabetes.³⁸ The progression of this condition can lead to sore formation which often can go unnoticed due to the numb effect.³⁸ The progression of the impairment due to diabetes results in sores that fail to heal and cause more damage as infection sets in.³⁸ The pathogenesis of neuropathy is closely associated with the progression of microvascular disease mentioned in detail under nephropathy.^{29,36} As explained in nephropathy, the high blood glucose levels affect the capillary basement membrane and create protein build up which narrows the blood vessels.³⁸ This process increases ROS productions which creates more damage and reduces the efficiency of the blood vessels.³⁶ Due to the damage of the blood vessels, treatment often fails to reach the site of treatment, thus leading to foot amputation in severe cases.³⁸ The Diabetes Control and Complications Trial study found that despite intensive control of glucose, there are other factors that influence in the evolution of the condition, thus early treatment of hyperglycaemia is vital.³⁸

1.4.3. Retinopathy

Diabetic retinopathy is a progressive disease that occurs in individuals who have had diabetes for prolonged periods. The prevalence of diabetic retinopathy is approximated at 40% for people with T1DM and 20% for those with T2DM.^{39,40} Retinol pericytes are specialized contractile cells that surround the retinol musculature and are crucial for blood flow regulation within the eye.^{41,42,43} Hyperglycaemia results in the destruction of the pericyte via osmotic damage, causing the capillary walls to weaken and blood flow to increase (due to decreased regulation within the retina).^{39,42,43} Focal dilations known as micro aneurysms form within the microvasculature, increasing the permeability of the membrane.^{39,42,43} With the high permeability, the proteins and lipids are released into the retina and form plaques known as exudates.⁴² The presence of the exudates affects the oxygen distribution, causing the tissue around the eye to die.^{42,43} In an effort to restore

the oxygen supply, the retina releases a vascular endothelial growth factor which increases vascularity (neovascularization) around the retina.⁴² Diabetic retinopathy results in individuals experiencing blurry vision which could progress to proliferative diabetic retinopathy that can cause complications such as retinal detachment or vitreous hemorrhage causing blindness.⁴²

1.5. Current treatments for type 2 diabetes mellitus

1.5.1. Western drugs

There are several antidiabetic medications available on the market for the management of T2DM. The most common treatments include:

- *Acarbose*: this drug inhibits the activity of α -glucosidase that is involved in the uptake and digestion of carbohydrates into the bloodstream. It also stimulates the synthesis and secretion of insulin.^{44,45} A common side effect associated with the use of acarbose is flatulence caused by the fermentation of unabsorbed carbohydrates.^{44,45}
- *Biguanides*: These drugs inhibit hepatic glucogenesis by increasing the uptake of glucose into peripheral cells, improving insulin sensitivity in musculature.^{44,45} Examples of biguanides include metformin and miglitol.^{42,43} Metformin is a relatively old antidiabetic drug with several undesirable side effects including hepatic and renal impairment, heart failure and anorexic effects.^{35,44} Miglitol's side effects include stomach pain, diarrhea and bloating.^{44,45}
- *Sulphonylureas*: This group of drugs stimulate insulin secretion by the β -cells of the pancreas, which reduces blood glucose levels over a short term.^{44,45} These drugs are not effective in managing long-term complications of the disease.^{44,45} A common side effect associated with usage of this drug is weight gain that may lead to hypertension.^{43,45} Examples of sulphonylureas currently used are gliclazide, glibenclamide, glimepiride, gliquidone and glipizide.^{44,45}

The side effects resulting from using Western medicine are undesirable to many people, and this is the reason why most diabetic patients search for alternative medication.³ Furthermore, the high cost of these treatment regimes also calls for the development of more affordable alternative medication.³

1.5.2 Traditional medicines

Traditional medicines of herbal origins have been used by humankind to treat a variety of ailments for eons.^{46,49} Written documents dating back 3500 years ago, describe the use of mandrake for pain relief by the Egyptians.⁴⁸ The Ayurvedic system that is still used medicinally, currently dates back several thousand years to a period called the Rig-Veda as known by Indians.⁵¹ The book *De Materia Medica* written in the first century AD was a long-standing book of medical reference to plant use in medicine in Europe.^{48,450} Herbal medicine use continued into the Middle Ages and the Renaissance with documents being efficiently kept by monasteries in areas such as England, Ireland and Germany.⁵¹ In the United States, there was an extensive usage of herbal medicine by the Native Americans, however little to no documentation is available.⁴⁶ European settlers colonized America and this is how documentation of traditional medicine use came about.⁴⁶ The early 1900s was the period when the world realized the potential for pharmaceutical industries and this was further supported by the technology advancements that focused on characterization of natural products, isolation, purification of compounds leading to synthetic chemistry.⁴⁶ The rise in synthetic chemistry resulted in a decline of herbal medicine use in developed countries as Western medicine was seen as the better option.⁴⁶

When studying the history or usage of traditional medicine in ancient Africa, not much is known, mainly due to poor documentation of this knowledge.⁴⁶ It was during the colonialism period where African traditional systems of medicine were highly challenged and the introduction of western medicine surfaced and became dominant.⁴⁷ Despite this, herbalists and traditional health practitioners (known as traditional healers) continued their practices and methodologies, which lead to the discovery and growth of various medicinal African plants offerings.⁵² Plants such as *Aloe ferox* (Cape Aloe), *Aloe vera*,

and *Aspalanthus linearis* (Rooibos tea), to mention a few, have seen constant growth of attention over the world due to their medicinal uses.⁵³

In 2015, the WHO reported that 80% of the African population uses traditional medicines for cultural practices and treatment of various public health diseases and health conditions.⁵ Similarly, many patients use traditional remedies to manage diabetes on the African continent.⁵⁴ During the introduction of insulin and synthetic oral hypoglycemic agents in the early twentieth century, a decline in traditional medicine was seen in developed countries.^{55,56} However, in recent years there has been growth in herbal remedy usage in both developing and developed countries, which includes for the treatment of diabetes.⁵⁷ Ethnopharmacological research has indicated the use of more than 1200 plants in the traditional medical systems targeted at treating hyperglycaemic activity.^{51,58} *In vitro* and *in vivo* studies have been carried out to evaluate these plant-based traditional medicine products.⁵⁹ The mechanism of action for most of these traditional plant-based products however still remains speculative.

1.6. Phytochemistry

Phytochemicals, also referred to as secondary metabolites, are defined as naturally occurring chemical compounds found in plant species. They have been found to be protective and preventative, in some cases, against many diseases and ailments.^{50,59} Phytochemicals offer protection from diseases and damage from the environment (pollution, stress, drought, UV exposure and pathogens).⁶¹ They are also responsible for the plant's colours, aroma and flavour.⁶¹ Phytochemicals accumulate in different parts of the plants such as the leaves, roots, stems, flowers, fruits or seeds.^{52,61} The distribution and concentration of phytochemicals tend to differ in different plant parts and also in the specific species growing under different environmental conditions.

Secondary plant metabolites offer antioxidant activity, antimicrobial effects, immune system stimulation, modulation of hormone metabolism and anticancer properties.^{59,60,61} Sugars, amino acids, proteins, chlorophyll and some nucleic acids form the primary

constituents, while phenolics, alkaloids, terpenoids, steroids, saponins, curcumin, glucosides form the secondary metabolites.⁶¹ According to literature, phenolics are the most abundant and structurally diverse plant phytoconstituents, followed by alkaloids.⁶¹

1.6.1. Phenolics

One way in which DM is managed is by alteration of a person's diet. It has been found the human diet allows for the ingestion of phytochemicals with therapeutic effects.^{62,63} Foods such as fruits and beverages have been found to contain phenolic compounds in limited amounts, however plants still remain the highest source of phenolics thus far.⁶³⁻⁶⁶ Food sources that have been shown to contain phenolics are: spices, fruits, seeds, and beverages such as coffee (200 – 500 mg of phenolics/cup), tea (150 – 200 mg of phenolics /cup) and wine (200 – 800 mg of phenolics/glass).⁶³⁻⁶⁵ New evidence has substantiated the health benefits of some cereals, cocoa products and olive oils due to the presence of phenolics.^{60,65}

Phenolics are hydroxy derivatives of aromatic carboxylic acids which have a single phenolic ring.^{60,61} Hydroxybenzoic acids and hydroxycinnamic derivatives form the main classes of phenolic acids found in edible plants.⁶⁰ Flavonoids are another form of phytochemicals which branch from phenolics that are abundant in the human diet.^{61,70} More than 400 varieties of flavonoids have been identified.⁶⁰

Oxidative stress as mentioned earlier, plays a significant role in initiation and progression of DM.⁹ The β -cells are subjected to pro-oxidation as they have a low concentration of antioxidant defence enzymes (SOD, CAT & GPx).⁶⁷ This is unfavourable for insulin producing cells as this leads to cell dysfunction that results in insulin resistance and decreased insulin secretion.⁶⁸ An increase in phenol intake in the diet or medication is ideal as they form some of the most potent antioxidants.^{69,70} The administration of antioxidants has been shown to increase the defence capacity of β -cells to oxidative stress.^{69,70}

1.6.2. Terpenoids

Terpenoids constitute the largest group of secondary metabolites.⁷¹ They are responsible for the fruit flavours, quality of agricultural products and also the fragrance released by flowers.⁷² They are also known as isoprenoids and consist of five groups; diterpenes, monoterpenes, sesquiterpenes, tetraterpenes and triterpenes.^{71,73}

The medicinal properties of these compounds vary depending on the source. Monoterpenes can offer analgesic and anti-inflammatory effects,⁷³ while diterpenes have been documented to possess anti-cancer and antihypertensive activity.⁷² Tetraterpenes which are found in vegetables such as carrots and pumpkin offer antioxidant activity,^{70,71} which may reduce the deleterious effects that ROS has on the β -cells.⁶⁸

1.6.3. Alkaloids

Plants containing alkaloids have been used since ancient times as a form of medicine, specifically as narcotics and stimulants. Modern Western diet consists of a large number of plants containing alkaloids and includes various vegetables and tea leaves.⁷⁴ These compounds are naturally occurring and are characterised by a heterocyclic ring structure containing a nitrogen atom.

More than 120 000 alkaloids have been identified.^{74,75} Alkaloids play a key role in biochemical mechanisms, physiological responses and have pharmacological effects.⁷⁵ Greg and colleagues, discovered that alkaloids (deoxynojirimycin, 2R,3R,4R,5R)-2,5-bis(hydroxymethyl)-3,4-dihydropyrrolidine) and flavonoids (Aloeresin A, Xanthones and Quercetin) have the ability to inhibit α -glucosidase activity thus preventing the breakdown of carbohydrates into monosaccharides.⁷⁵ The transport of glucose to the intestinal epithelium is also decreased, which results in a decrease in blood glucose levels.⁷⁵

Natural products have shown great potential as antidiabetic agents and also have the potential to tackle various mechanisms of action which are crucial in diabetes progression.^{65,66} Various of the plants, used to treat diabetes, investigated by Tabatabaei

et al. were found to reduce oxidative stress and improve β cell function, thereby promoting insulin secretion.⁶⁵

1.7. Medicinal plants investigated in this study

The two plant species investigated in this study are used traditionally to treat diabetes, *Gnidia canoargentea* and *Osyris lanceolata*. These plant species were selected after consultation with a Traditional Health Practitioner (THP) from North West province, South Africa, who approached the Council for Scientific and Industrial Research (CSIR) requesting the investigation of the plant species for diabetes treatment and management, for which he employs them. There was no record found reporting the use of the two plant species in managing diabetes.

1.7.1. *Gnidia canoargentea* (C.H. Wright) Gilg

Gnidia canoargentea commonly known as the Highveld silver yellow head belongs to the Thymelaeaceae family (Figure 5).⁷⁶ It is an undershrub plant, arising from a woody base.⁷⁷ It is characterized by a smooth woody textured bark with lenticels.⁷⁷ The branches are slender and alternate with conspicuous flowers near sessile leaves.⁷⁷ It is indigenous to South Africa and grows in the Free State, Gauteng, Limpopo, Mpumalanga and North West provinces.⁷⁶



Figure 5: *Gnidia canoargentea* A) whole plant, B) flower, leaves and stem (from SANBI Red List).

G. canoargentea is one of 150 species of *Gnidia*.⁷⁸ Of the species, 50% are located in the northern parts of South Africa, with some spreading to other parts of Africa and a few being found in Madagascar, India and Sri Lanka.⁷⁸

While there is no record of using *Gnidia* species for metabolic disorders or diabetes, there have been various medicinal uses reported for the genus. The *Gnidia* species is currently used to treatment of heart problems, chest problems, and stomach issues,⁷⁹ treat fever, assist women to conceive/give birth,⁴⁰ ease asthma, back pain, headaches, and nightmares.⁸⁰ They are also used to treat epilepsy, tuberculosis, snakebites, tonsillitis and dropsy.^{79,80}

1.7.2. *Osyris lanceolata* (Hochst & Steud)

Osyris lanceolata (synonymous to *Osyris quandripartita*) commonly known as African sandalwood, belongs to the family Santalaceae (Figure 6). It is an evergreen, hemiparasitic tree that can grow 2 - 6 m high.⁸¹ It is characterized by a smooth, dark-brown leather bulk and blue greenish leaves.⁸¹ This plant is native to Kenya, Tanzania and South Africa. It is found in half of the Eastern parts of South Africa and the central

parts of Africa.⁸¹ It grows well on rocky mountain slopes in the bushveld, wooded forest and grasslands.⁸¹



Figure 6: *Osyris lanceolata* A) whole plant B) leaves and stem of plant (Tropical.theferns.info)

The *Osyris* genus consists of approximately 34 species, some of which are partially parasitic.⁸³ Plants under this genus are found in northern Africa, southwest Asia and southern Europe.⁸³ *O. lanceolata* roots have been used traditionally to treat venereal diseases, infertility, menorrhagia and styptic (helps close open wounds quicker) effects on wounds by various African tribes in Tanzania and Kenya.⁸⁴ There were no studies or records regarding the use of the *Osyris* plant species for diabetic or metabolic disorders.

1.8. Aim and objectives

The aim of this study was to assess the effects of medicinal plants *G. canoargentea* and *O. lanceolata* in regulating glucose uptake using *in vitro* biological tests. The carbohydrate hydrolyzing enzymes and metabolic disorders such as oxidative stress and insulin action are considered to be at the center of diabetes condition and progression.

The objectives of the study were to:

- identify the phytochemical classes present in the plant extract using thin layer chromatography and ultra-performance liquid chromatography.
- analyse the potential of the plant extracts to reduce hyperglycaemia by inhibition of enzyme activity α -amylase and α -glucosidase.
- assess the potential of the selected plants to promote glucose uptake in C2C12 cells using the 2-[N-(7-nitrobenz-2-oxa-1,3-dioxol-4-yl) amino]-2-deoxyglucose assay.
- assess the potential of the plant species to act as antioxidants, using the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-2-picrylhydrazyl (DPPH) assays.
- determine the cytotoxicity of the plant species using the sulphorhodamine B (SRB) assay and two cell lines (C2C12 mouse myoblasts and Ea.hy926 endothelial cells).

1.9. Project outline

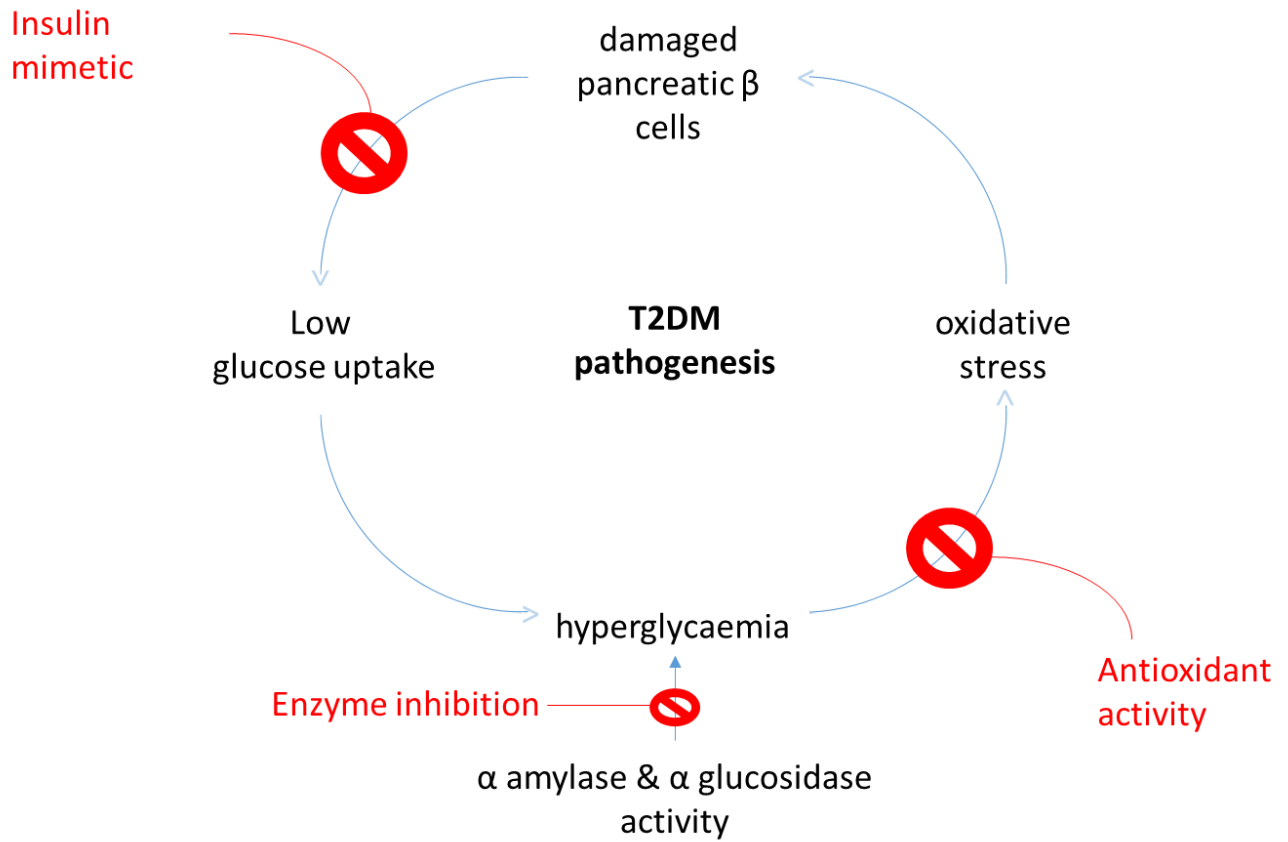


Figure 7: Schematic outline of the project.

Chapter 2: Materials and methods

Ethics approval to conduct this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences, University Pretoria (Appendix I). All reagents used and the preparation thereof is provided in the Appendix II.

2.1. Plant material

This study is based on the prescription of *G. canoargentea* and *O. lanceolata* for the treatment of diabetes in North West province, South Africa by a THP. The plants were collected by the THP and supplied to the CSIR under the Material Transfer Agreement . This agreement stipulates the conditions for the utilisation of the plant species through scientific research. The specimens were sent to the South African National Biodiversity Institute (SANBI, Tshwane), for botanical identification and verification. Voucher specimens are deposited at SANBI.

2.2. Extract preparation

The leaves and stems of the plants were air dried in the dark and ground to a fine powder using a hammer mill grinder and stored in air tight containers at the CSIR processor plant. To mimic the ethnomedicinal preparation by the THP, the combination mixture consisted of 60 g of *O. lanceolata* and 40 g of *G. canoargentea* in 1 L dH₂O. In addition, aqueous extracts of single plants were prepared as follows; plant material (50 g) was added to 500 mL distilled water (dH₂O) and brought to boil for 15 min while stirring. After boiling, extracts were left to cool, then filtered before freezing at -20°C in preparation for freeze-drying.

For the preparation of extracts to simulate pharmaceutical preparations, organic extracts of single plants were prepared. This was done by adding 100 g of the material to 1 L of

dichloromethane (DCM): methanol (1:1). The mixture was left on a shaker at room temperature for 24 h, after which it was filtered through a Whatman No. 1 filter paper (the process was repeated three times). The filtrate was combined and concentrated with a vacuum rotary evaporator (Heidolph instrument) at 60°C. The dried samples were stored at -20°C until analysis. From the DCM:MeOH organic extract, 50% of sample was used to prepare a compound concentrated chloroform partition using water and chloroform.

Extract yields were determined gravimetrically using the equation below:

$$\% \text{ Gravimetric yield} = W_s \times \frac{V}{W_d} \times 100$$

Where,

W_s : weight of extract solubles in one mL of extract solution (mg/mL)

W_d : starting dry weight (mg)

V : volume of solvent used for extraction (mL)

2.3. Phytochemical analysis

2.3.1. Thin layer chromatography

Thin layer chromatography (TLC) was employed to identify which major phytochemical classes (alkaloids, flavonoids, phenolics, saponins, sterols, terpenoids) were present in the organic plant extracts. Pre-coated silica plates (Merck, Sil G-25 UV 254, 20 cm x 20 cm) and the reagents specified in Table 1 were used to detect compounds.

Table 2: Mobile phases and spray reagents used in the detection of phytochemical classes using thin layer chromatography.

Phytochemical class	Mobile phase	Spray reagent (Mixture)
Alkaloids	Acetone:chloroform:methanol (4:4:2)	Dragendorff reagent
Flavonoids	Acetone:chloroform:methanol (4:4:2)	3% Sodium nitrate, 1% Aluminium chloride
Phenolic acids	Ammonium hydroxide:methanol (3:200)	Folin-Ciocalteu
Saponins	Chloroform:methanol: water (60:35:5)	1% Vanillin, 5% Sulphuric acid
Sterols	Chloroform:methanol (3:4)	85% Phosphoric acid: water
Terpenoids	Toluene:ethyl acetate (93:7)	1% Vanillin, 5% Sulphuric acid

2.3.2. Ultra-performance liquid chromatography

A Waters Acquity UPLC coupled in tandem to a Waters SYNAPT HDMS G1 mass spectrometer was used to generate chromatographic and mass spectral data. Optimization of the chromatographic separation was done utilizing a Waters Acquity HSS T C18 column (150 × 2.1 mm; 1.8 µm). The column was heated to 60°C. A binary solvent mixture was used with solvent A consisting of 0.1% (v/v) formic acid in Milli-Q water and solvent B consisting of 0.1% (v/v) formic acid in acetonitrile. The initial conditions were 98% A at a flow rate of 0.4 mL/min, which was maintained for 1 min, followed by a linear gradient to 5% A at 25 min. These conditions were maintained for 2 min then changed back to the initial conditions. The total run time was 30 min. The injection volume was 2 µL.

The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray ionization mode to enable detection of electron spray ionization (ESI) compatible compounds. Leucine enkephalin (50 pg/mL) was used as reference calibrant to obtain typical mass accuracies between 1 and 5 mDa. The mass spectrometer was operated in the ESI positive and negative mode with the sampling cone set at 30 V, extraction cone at 4 V and a capillary voltage of 2.5 kV. High purity nitrogen gas was used as a nebulization gas at flow rate of 550 L/h and cone gas was added at 50 L/h. The desolvation temperature was set at 450°C. The scan time was 0.1s covering a 50 to 1500 Da mass range. The hyphenated system was controlled with MassLynx Ver. 4.1 (SCN 872) software and was used for all data manipulation.

2.4. Enzyme activity assays

2.4.1. α -amylase

The determination of α -amylase inhibition was carried out according to Bernfeld *et al.*⁸⁵ Pancreatic α -amylase initiates hydrolysis of the 1,4 α -glucosidic bonds of long chained carbohydrates to yield reducing sugars. The presence of the reducing sugar is monitored using 3,5-dinitrosalicylic acid (DNSA) which is initially yellow in colour and is reduced to 3-amino-5-nitrosalicylic acid (ANSA) that is orange-red in colour. The reaction was monitored using a spectrophotometrically.

A porcine pancreatic α -amylase (Sigma-Aldrich, St Louis, USA) stock solution (0.2 U/mL) was prepared in sodium phosphate buffer (9.66 mM, pH 6.9) and stored on ice for the duration of the experiment. A 0.5% (w/v) potato starch solution prepared in 50 mL phosphate buffer was used as the substrate and acarbose (0.625 – 20 μ g/mL in reaction) as the positive control. Into a 96-well plate, 30 μ L organic plant extract (25-100 μ g/mL in reaction), 60 μ L potato starch and 30 μ L of porcine pancreatic α -amylase (2 U/mL) was pipetted and the plate incubated at 25°C for 5 min. After incubation, 60 μ L of colorimetric reagent (DNSA, Sigma-Aldrich, St Louis, USA) was added before the 96-well plate was

placed in a water bath (85°C) for 10 min. The absorbance was measured at 540 nm and the α -amylase inhibitory activity was determined using the following equations:

$$\text{End Point Enzyme Activity (\%)} = \frac{[\text{starch (test)}]}{[\text{starch (control)}]} \times 100$$

$$\text{Inhibition (\%)} = 100 - \text{enzyme activity (\%)} \pm \text{SEM}$$

2.4.2. α -glucosidase

The α -glucosidase activity was assessed using the method described by Kim *et al.*⁸⁶ and Sancheti *et al.*⁷² The assay works on the principle that α -glucosidase breaks down the substrate, 4-nitrophenyl α -D-glucopyranoside, into α -D-glucose and 4-nitrophenol. The colorimetric product 4-nitrophenol is then measured spectrophotometrically.

The α -glucosidase solution was prepared by dissolving 0.5 g rat intestinal acetone powder (Sigma-Aldrich, St Louis, USA) in 10 mL of 0.9% saline which was sonicated for 5 min in an ice bath and centrifuged (5000 *g*, 30 min, 4°C). Into a 96-well microplate, 30 μ L of the plant extract (25 - 100 μ g/mL in reaction), 30 μ L of the rat intestinal α -glucosidase supernatant solution (0.1 M, pH 7) and 35 μ L of phosphate buffer (0.1 M, pH 7) was added. The reaction was incubated for 10 min at 37°C. Thereafter, 30 μ L of substrate (0.5 mM 4- nitrophenyl α -D-glucopyranoside (Sigma-Aldrich, St Louis, USA) was added to the reaction mixture and incubated at 37°C for 30 min. Acarbose was used as positive control (25 - 100 μ g/mL). The absorbance was measured at 405 nm and the α -glucosidase activity calculated using the equations bellow:

$$\text{End Point Enzyme Activity (\%)} = \frac{[4 - \text{nitrophenol (test)}]}{[4 - \text{nitrophenol (control)}]} \times 100$$

$$\text{inhibition (\%)} = 100 - \text{reaction rate (\%)} \pm \text{SEM}$$

2.5. Cell culture and maintenance

The C2C12 mouse myoblast (ATCC CRL-1772) cell line was purchased from the American Type Tissue Culture Collection. The cells were cultured in a 75 cm³ cell culture flask and maintained in Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, USA), supplemented with 1% penicillin/streptomycin and 10% foetal calf serum (FCS) (PAA, Pasching, Austria) at 37°C in an incubator with 5% atmospheric CO₂. The differentiation of C2C12 myoblasts into myotubes was done according to the method described by Burattini *et al.*⁸⁷ The myoblasts were transferred to a 75 cm³ cell culture flask. Differentiation medium consisting of DMEM with 1% FCS was added and the cell left to differentiate over 7 days (the medium was changed every 24 h) in an incubator set at 37°C with 5% atmospheric CO₂. The differentiation of the C2C12 (formation of multinucleated myotubes) was confirmed using phase contrast microscopy before trypsinization and seeding into 96 well plates.

The endothelial cell line, Ea.hy926, was gifted by Dr C Edgell. Cells were cultured in 75 cm³ cell culture flasks and maintained in DMEM, supplemented with 1% penicillin/streptomycin and 10% FCS in an incubator set at 37°C with 5% atmospheric CO₂.

The cells were grown to 80% confluence, washed with PBS and chemically detached using trypsin (The Scientific Group, South Africa) for Ea.hy926 cells and TrypLE (The Scientific Group, South Africa) in EDTA for C2C12 cells. The detached cells in solution were centrifuged at 200 *g* for 5 min each and the pellets re-suspended in 1 mL medium. To determine cell density, cells were counted using the trypan blue exclusion assay. The remaining cells were re-suspended in 10% FCS supplemented medium.

2.6. Cytotoxicity evaluation assay

Cytotoxicity was assessed using the sulphorhodamine B (SRB) assay as described by Vichai and Kirtikara.⁸⁸ Cells (100 μ L) were seeded at a concentration of 1.0×10^4 cells/well for C2C12 and 1.5×10^4 cells/well for Ea.hy926 cells. Cells were allowed to attach by incubating for 24 h before being exposed to extracts. The blank wells contained 200 μ L DMEM supplemented with 5% FCS. To specific wells were added; 100 μ L plant extract (0.02- 100 μ g/mL in reaction), 0.5% DMSO (vehicle control), 1% saponin (Sigma Aldrich, St Louis, USA) (positive control) or 100 μ L FCS non supplemented DMEM (negative control) and plates incubated for 24 h. Following incubation, 100 μ L cold trichloroacetic acid (TCA, 50%) (Merck Chemical Company, Darmstadt, Germany) was added to fix the cells, followed by an incubation period of 24 h at 4°C. After fixation, plates were washed three times with tap water and left to dry at 40°C. The plates were stained by adding SRB (0.057%, 100 μ L) (Sigma Aldrich, St Louis, USA) to each well and left for 30 min. The excess dye was removed by rinsing three times with 100 μ L acetic acid (1%) and the plates were left to dry at 40°C. Trisaminomethane (TRIS) base solution (200 μ L, 10 mM, pH 10.5) was utilized to solubilize the bound SRB and shaken for 30 min, after which the absorbance was measured at 510 nm (reference wavelength 630 nm) using a microplate reader (Synergy 2, Bio-Tek instrument Inc). Cell density was determined using the equation:

$$\begin{aligned} & \text{Cell Density (\% of negative control)} \\ &= \left(\frac{\text{absorbance of sample}}{\text{average absorbance of negative control}} \right) \times 100 \end{aligned}$$

2.7. *In vitro* antioxidant activity assays

2.7.1. Azinobis-3-ethylbenzothiazoline-6-sulfonic acid assay

The cell-free antioxidant assay was carried out as described by Re *et al.*⁵⁶ with minor modifications. The ABTS solution was prepared by adding ABTS⁺ solution (7 mM) to 2.45

mM potassium sulfate ($K_2S_2O_8$) in dH_2O and incubating in the dark at $4^\circ C$. The ABTS solution was diluted with dH_2O to a standard absorbance of 0.7 ± 0.02 at 734 nm prior to experimentation. The positive control wells contained Trolox (1-10 $\mu g/mL$) (Sigma-Aldrich, St Louis, USA) dissolved in dH_2O by sonication, whereas the negative control consisted of $ABTS^{\cdot+}$ in dH_2O . Distilled water served as the blank. Into the wells of a 96-well plate was added, 15 μL extract (3.125-100 $\mu g/mL$ in reaction) and 200 μL of $ABTS^{\cdot+}$ solution. The plate was incubated at room temperature in the dark for 15 min before being spectrophotometrically read at 630 nm. The antioxidant activity was calculated using the following equation:

$$Scavenging\ activity\ (\%) = \frac{A(control) - A(extract)}{A(control)} \times 100$$

$A(control)$ = absorbance of control

$A(extract)$ = absorbance of extract

2.7.2. Diphenyl-2-picrylhydrazyl assay

The diphenyl-2-picrylhydrazyl (DPPH) scavenging activity was performed following the method described by Gyamfi *et al.*⁷¹ with minor modifications. The DPPH solution was prepared fresh for every experiment by sonicating 240 μM DPPH in pure MeOH for 20 min. Into a 96-well plate, 20 μL of organic plant extract (3.125 - 100 $\mu g/mL$ in reaction) and 180 μL DPPH solution was added. The control wells contained distilled water (negative control) or Trolox (1-10 $\mu g/mL$) as a positive control (Sigma-Aldrich, St Louis, USA). Thereafter, the plate was incubated for 15 min in the dark at room temperature. The reaction was monitored spectrophotometrically at 570 nm using a microplate reader (Synergy 2, Bio-Tek Instruments Inc.). The results were expressed quantitatively using the same equation used for the $ABTS^{\cdot+}$ assay.

2.7.3. Intracellular reactive oxygen species determination

The dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay was used to monitor the attenuation of AAPH-induced ROS by the plant extracts as described by Boissy *et al.*⁵⁸ with minor modifications. The permeable fluorogenic probe DCFH-DA (Sigma-Aldrich, St Louis, USA) when in the cell is deacetylated by esterase and activated by ROS into a highly fluorescent product, dichlorodihydrofluorescein (DCF). Trolox was used as a positive control and *p*-chloranil (Sigma-Aldrich, St Louis, USA) in DMEM as negative control.

To pre-seeded white 96-well microplates 100 μ L medium containing DCFHDA (5 μ M) was added in each well. The plates were incubated for 1 h at 37°C and 5% CO₂. Thereafter the DCFHDA medium was aspirated and 100 μ L of plant extract (3-100 μ g/mL in reaction) or 20 μ g/mL Trolox, or 100 μ L medium in a control. A volume of 100 μ L of AAPH (40 μ M in reaction) was added in all wells and plates incubated for 3 h. The fluorescence was measured at λ_{ex} = 485 nm and λ_{em} = 540 nm (FLUOsta Optima, BMG Labtech) and expressed in terms of relative fluorescence intensity (RFI).

$$RFI = \frac{Test - Blank}{control - blank} \times 100$$

2.8. Glucose uptake

The 2-[N-(7-nitrobenz-2-oxa-1,3-dioxol-4-yl) amino]-2-deoxyglucose (2-NBDG) is a fluorescent analogue of 2-deoxyglucose and can be used for directly monitoring insulin-mediated glucose uptake into myotubes. If a compound exhibits insulin mimetic effects, the rate of 2-NBDG uptake is increased.

Glucose uptake was assessed using the assay described by Zou *et al.*⁵⁹, with minor modifications. C2C12 cells (1.0 x 10⁴ cells/well) were plated into a 96-well and incubated for 24 h at 37°C in 5% CO₂. Thereafter, the cells were exposed to 50 μ L of cell culture

medium containing 400 μM of fluorescent 2-NBDG (Invitrogen Molecular probe, USA) together with 80 μL plant extract (3-100 $\mu\text{g/mL}$ in reaction). Insulin was used as positive control (80 μL of 20 $\mu\text{g/mL}$ in reaction). Cells treated with medium containing fluorescent 2-NBDG (100 μL) served as negative control. The plates were incubated at 37°C in 5% CO_2 for 1 h. The 2-NBDG uptake reaction was stopped by removing the medium where after, the cells were washed with cold PBS. Cells in each well were suspended in 100 μL PBS and the plate was read at $\lambda_{\text{ex}} = 460 \text{ nm}$ and $\lambda_{\text{em}} = 544 \text{ nm}$ and expressed in terms of RFI.

$$RFI = \frac{\text{Test} - \text{Blank}}{\text{control} - \text{blank}} \times 100$$

2.9. Statistical analysis

All experiments were carried out on three occasions and in triplicate ($n \geq 9$). Data collected was compiled using Microsoft Excel 2016 and analysed with GraphPad Prism 6.0. The data is expressed as mean $\pm$ standard error of the mean (SEM). Statistical significance between control and sample were assessed with the Dunn's test ($P < 0.05$).

Chapter 3: Results and discussion

3.1. Phytochemical screening

3.1.1. Extract yield and chromatographic analysis

Extracts of *O. lanceolata* and *G. canoargentea* were made using water and DCM:MeOH. The yields of extracts per plant species and in combination are indicated in Table 3.

Table 3: Gravimetrically determined mean percentage of the aqueous and organic solvent extracts yields

Plant species	Extract yields (%)	
	Aqueous extract	DCM-MeOH extract
<i>G. canoargentea</i>	14.62	38.66
<i>O. lanceolata</i>	8.36	27.44
Combination of the plant species	27.65	-

- = the combination of the plant extract was done as per the THP's method of using water only

Based on TLC plate analysis, both plant species were found to contain the phytochemical classes tested for, except for the alkaloids which were not detected (Table 4).

Table 4: Phytochemical analysis of the DCM-MeOH extracts of plants by TLC chromatography.

Phytochemical class	<i>O. lanceolata</i>	<i>G. canoargentea</i>
Alkaloids	-	-
Flavonoids	+	+
Phenolic acids	+	+
Saponins	+	+
Sterols	+	+
Terpenoids	+	+

+: presence of the phytochemical class

-: absence of the phytochemical class

The results obtained for *O. lanceolata* (also known as *Osyris quandripartita*) with respect to the presence of flavonoids in the DCM:MeOH extract, is corroborated by Girma *et al.*⁹¹ The absence of alkaloids in *O. lanceolata* is contradictory to a previous report by Shyaula which identified the presence of pyrrolizidine, pyrimidine and quinolizidine alkaloids in the plant species.⁹² This contraction in the presence of the phytochemical in the same plant species may be attributed to the geographic location, weather patterns and the soil type, as these have been shown to have an impact in the chemical fingerprinting of plant species. In this study, the plant species was sourced from North West province in South Africa.¹³⁵⁻¹³⁷

Using UPLC-MS analysis, the presence of 6-hydroxyflavone, perlagonidin-3-O-glucoside and perlargonidin-3-O-rutinoside were tentatively identified in *O. lanceolata* (Table 5). The UPLC-MS chromatograms are depicted in Figures 7 to 9. The chemical compounds are categorized within the flavonoid class, and thus support the results obtained after TLC analysis (Table 4). Perlagonidin-3-O-rutinoside and perlagonidin-3O-glucoside are phenolic compounds that are commonly found in berry fruits such as strawberries and mulberries and has been shown by liquid chromatography-mass spectrometry to exhibit a mass of 579 m/z and 433 m/z respectively.^{93,94}

Table 5: Phytochemical compounds detected in *G. canoargentea* and *O. lanceolata* DCM:MeOH, water extracts and chloroform partition using UPLC-MS.

Plant	Compound	Solvent fraction	Retention time (minutes)	Chemical formula	UPLC-MS(ESI)	
					[M+H] ⁺	[M+H] ⁻
<i>G. canoargentea</i>	Kaempferol-3-O-rutinoside	DCM:MeOH	12.90	C ₂₇ H ₃₀ O ₁₅	593.166	-
		H ₂ O	12.90			
		Chloroform	19.17			
	Rutin	DCM:MeOH	11.94	C ₂₇ H ₃₀ O ₁₆	-	609.1456
		H ₂ O	11.94			
		Chloroform	11.72			
<i>O. lanceolata</i>	6-Hydroxyflavone	DCM:MeOH	17.21	C ₁₅ H ₁₀ O ₃	239.0708	-
		H ₂ O	17.18			
		Chloroform	17.21			
	Pelargonidin-3-O-glucoside	DCM:MeOH	14.52	C ₂₁ H ₂₁ O ₁₀ ⁺	433.1135	-
		H ₂ O	14.52			
		Chloroform	14.52			
	Pelargonidin-3-rutinoside	DCM:MeOH	14.69	C ₂₇ H ₃₁ O ₁₄ ⁺	579.1594	-
		H ₂ O	14.69			
		Chloroform	16.17			

Two flavonoids, namely kaempferol-3-O-rutinoside and rutin were tentatively identified in both extracts of *G. canoargentea* using UPLC-MS (Figure 11 and 12). There is limited literature reporting the phytochemical analysis of *G. canoargentea*, however several species (such as *Gnidia glauca*) of the *Gnidia* genus have been reported to have the presence of phenolic acids in the stems and flavonoids in the flowers of the plant species when extracted using methanol.⁹¹ The leaves, stem and flowers of *G. canoargentea* used to acquire the extracts in the present study also yielded phenolic and flavonoid compounds even though different solvent systems were used. Thus, it can be deduced that the plant part plays a role on the phytochemical make-up of a plant species.

Parejo *et al.*, found kaempferol-3-O-rutinoside to be present in *Foeniculum vulgare* water extract and showed an ionization mass of 593, which is congruent to the ionization mass found in this study; however, the retention time differed by 5 min.⁹⁵ Furthermore, the study found rutin (also known as quercetin-3-O-rutinoside) to have an ionization mass of 609 with a retention time of 16.80 min, which was also confirmed during analysis in the present study.⁹⁵ The time difference observed in the retention times of kaempferol-3-O-rutinoside and rutin between the current and previous study could be ascribed to the different HPLC methods used.

3.2. Enzyme activity

Modulation of intestinal carbohydrate digestion is one way in which T2DM can be controlled. Inhibition of carbohydrate hydrolyzing enzymes such as α -amylase and α -glucosidase can assist in decreasing post-prandial hyperglycaemia, which is a major pathological contributor to the progression of the disease.⁹⁶

Currently, acarbose is one of the drugs used to modulate carbohydrate digestion. Acarbose reduces the glucose absorption rate thereby lowering post-prandial blood glucose levels. However, acarbose has various side effects which include flatulence, liver disorders and diarrhoea.⁹⁷ There is a growing need to identify natural substitutes for managing T2DM with the hope of avoiding the medicinal side effects caused by the current Western drugs or medicine. In this study, the single plant water extracts of *G. canoargentea* and *O. lanceolata* did not inhibit α -amylase and α -glucosidase activity (Figures 13 and 14); this was also true for the plant combination extract with regards to α -amylase activity (Figure 13G). The DCM:MeOH extract of *O. lanceolata* (Figure 13B) had a plateaued effect, which may suggest saturation of the enzyme with the plant extract (substrate) as an active inhibitor. This warrants for further research of how the enzyme reacts at lower plant extract concentrations so as to ascertain the most effective concentration to use.

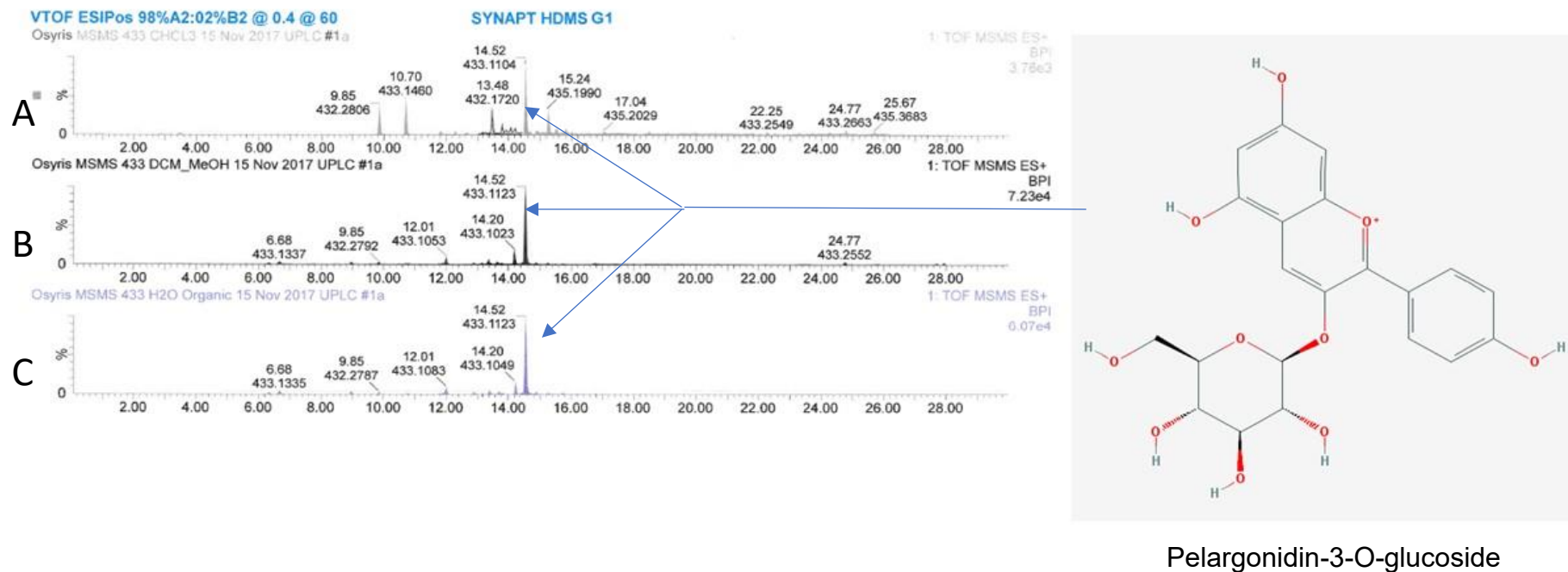


Figure 8: UPLC-TOF-MS chromatograms indicating the presence of pelargonidin-3-O-glucoside in A) chloroform, B) DCM:MeOH and C) water extract of *O. lanceolata*.

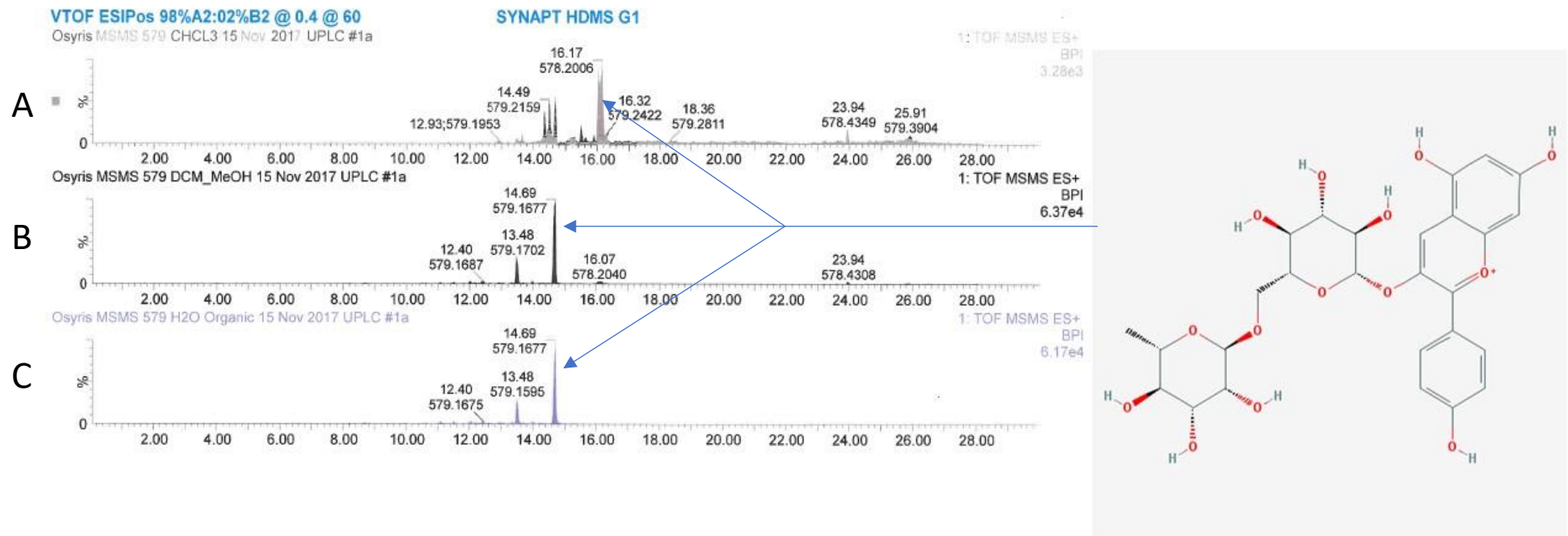


Figure 9: UPLC-TOF-MS chromatograms indicating the presence of pelargonidin-3-O-rutinoside in A) chloroform, B) DCM:MeOH and C) water extract of *O. lanceolata*.

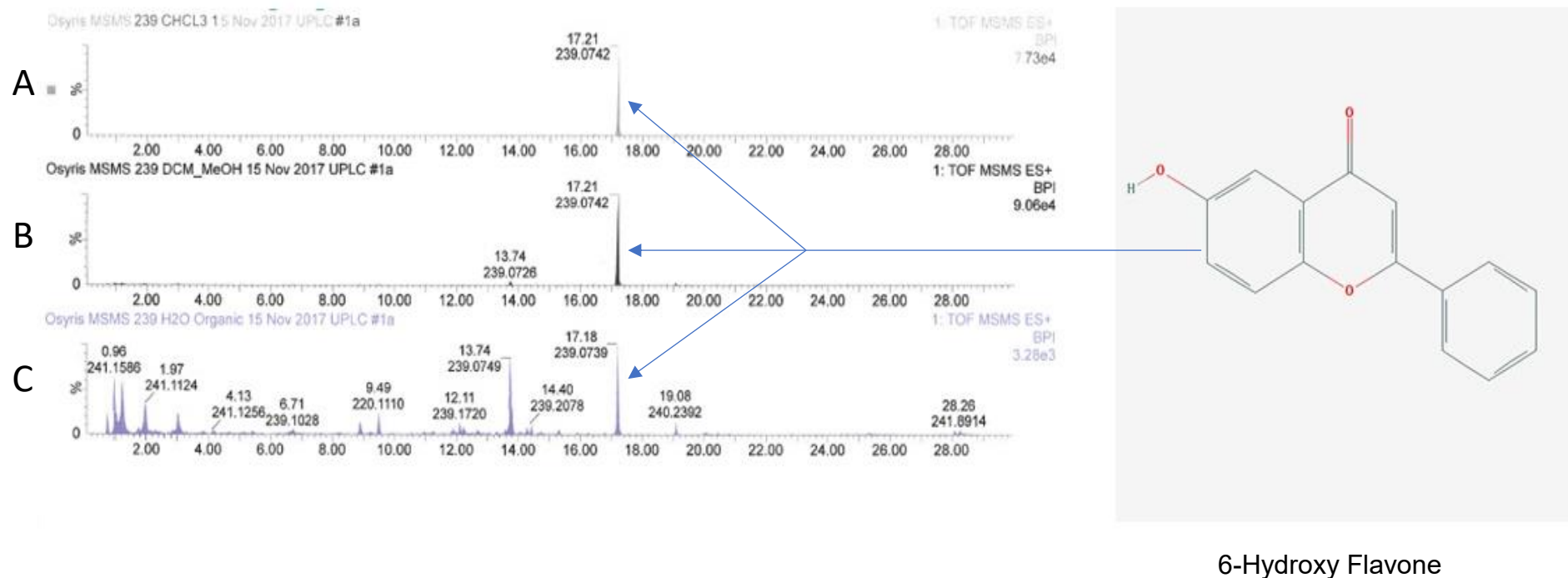
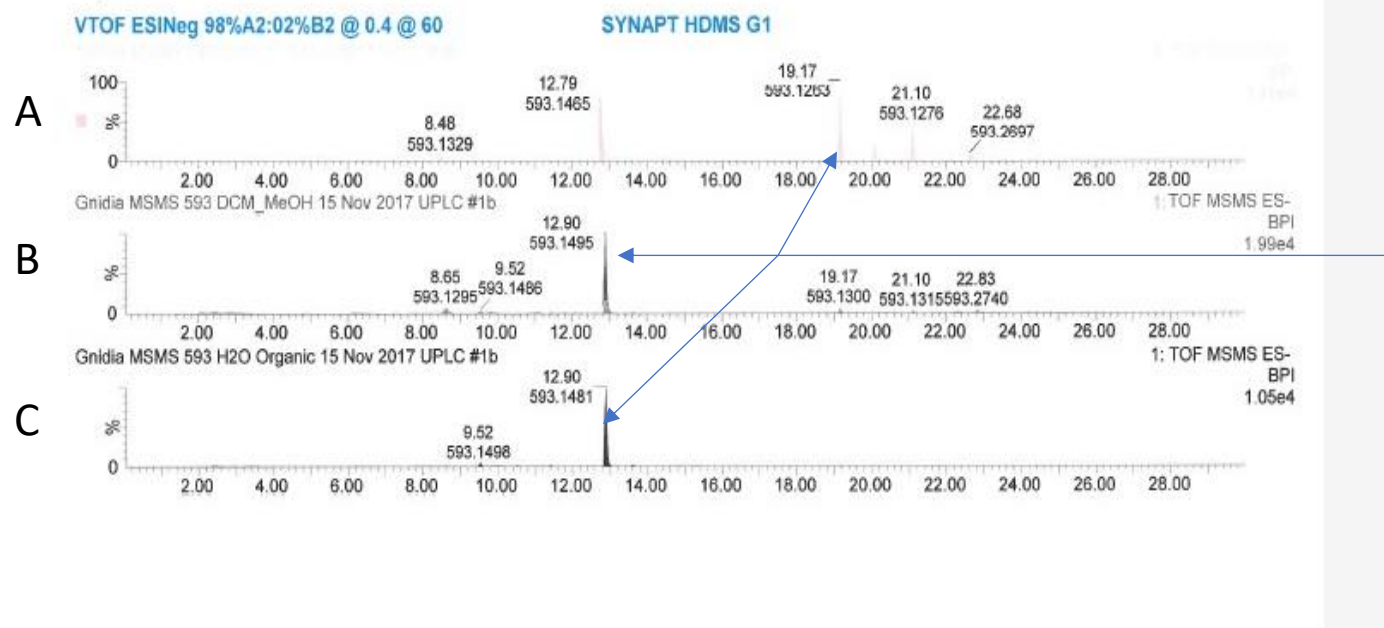


Figure 10: UPLC-TOF-MS chromatograms indicating the presence of 6-hydroxy flavone in A) chloroform, B) DCM:MeOH and C) water extract of *O. lanceolata*.



Kaempferol-3-O-rutinoside

Figure 11: UPLC-TOF-MS chromatograms indicating the presence of kaempferol-3-O-rutinoside in A) chloroform, B) DCM:MeOH and C) water extract of *G. canoargentea*.

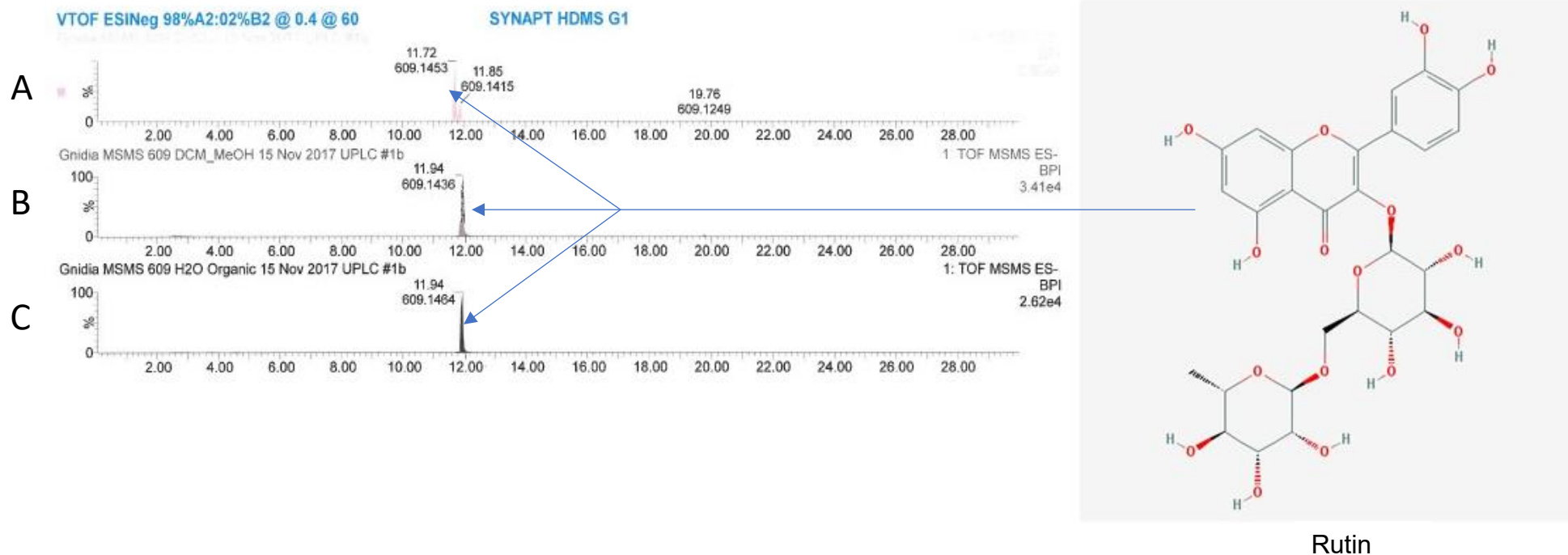


Figure 12: UPLC-TOF-MS chromatograms indicating the presence of rutin in in A) chloroform, B) DCM:MeOH and C) water extract of *G. canoargentea*.

The water extract of *G. canoargentea* had a scattered inhibitory profile (Figure 13D), this effect may be a result of the extract colour of which a colour control could help address. The chloroform partition of *O. lanceolata* with the DCM: MeOH extracts of *G. canoargentea* and the chloroform partition of *O. lanceolata* (Figure 12) resulted in a concentration dependent inhibitory effect against α -amylase. In the case of α -glucosidase, all the extracts resulted in a concentration dependent inhibitory effect, however the inhibition was minimal and below 50%. It was also found that all plant extracts samples (except for *O. lanceolata* water extract) at low concentrations (3.125 $\mu\text{g/mL}$ and 6.12 $\mu\text{g/mL}$) exhibited negative values with regards to α -glucosidase enzyme inhibition. This activity could suggest that the plant extract at low concentrations may increase the activity of the enzyme rather than inhibit it. Further probing into the observed effect is thus needed so as to confirm this activity.

Moreover, the results showed that both plant species had an individual inhibitory effect on α -amylase, but to a limited extent. *G. canoargentea* exhibited a more pronounced inhibitory activity than *O. lanceolata* on α -amylase. The IC_{50} values could only be obtained for the *G. canoargentea* DCM:MeOH (Figure 13E) and the chloroform partition extracts (Figure 13F); however these extracts were not found to be as effective as the positive control acarbose ($\text{IC}_{50}=8.33\mu\text{g/mL}$). UPLC-MS analysis provisionally indicated the presence of the anthocyanins, pelargonidin-3-O-glucoside and pelargonidin-3-O-rutinoside within the *O. lanceolata* extracts. These two compounds have been reported to have α -glucosidase inhibitory effects from mulberry fruit *in vitro* using Spearman's correlation coefficient, where pelargonidin-3-O-glucoside had a correlation of 0.760 and pelargonidin-3-O-rutinoside at 0.883 conficiency.⁹⁸ Pelargonidin-3-rutinoside was found to have an α -glucosidase inhibition of $\text{IC}_{50}= 64.5 \mu\text{g/mL}$.⁹⁹ Kaempferol and rutin found to be present in the *G. canoargentea* extracts also share similar properties to the anthocyanins, in that they inhibit α -glucosidase.^{95,60}

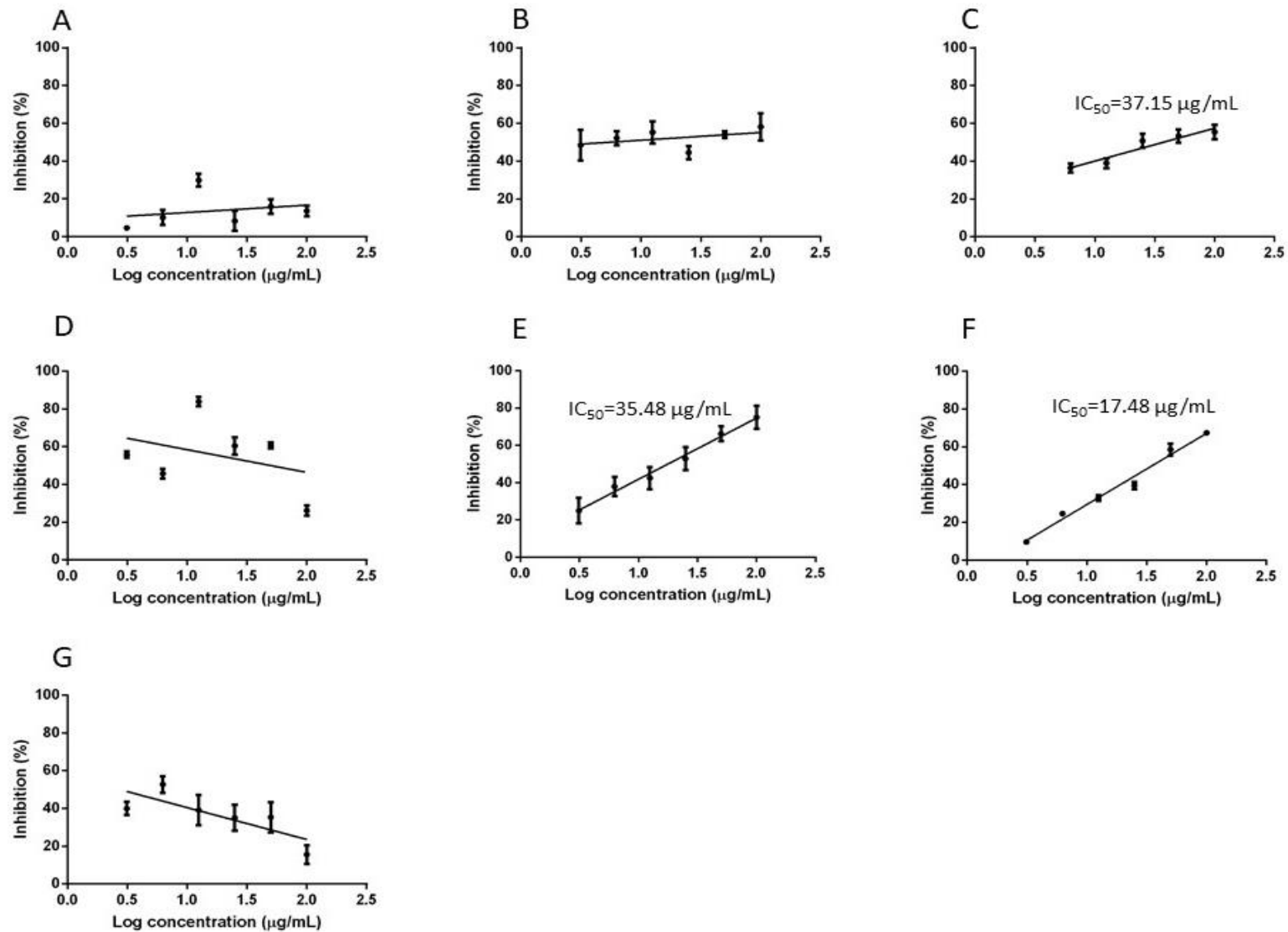


Figure 13: The effect of plant extracts on α -amylase activity. A) Water extract of *O. lanceolata*, B) DCM:MeOH extract of *O. lanceolata*, C) chloroform partition extract of *O. lanceolata*, D) water extract of *G. canoargentea*, E) DCM:MeOH extract of *G. canoargentea*, F) chloroform partition of *G. canoargentea* and G) combination plant extract.

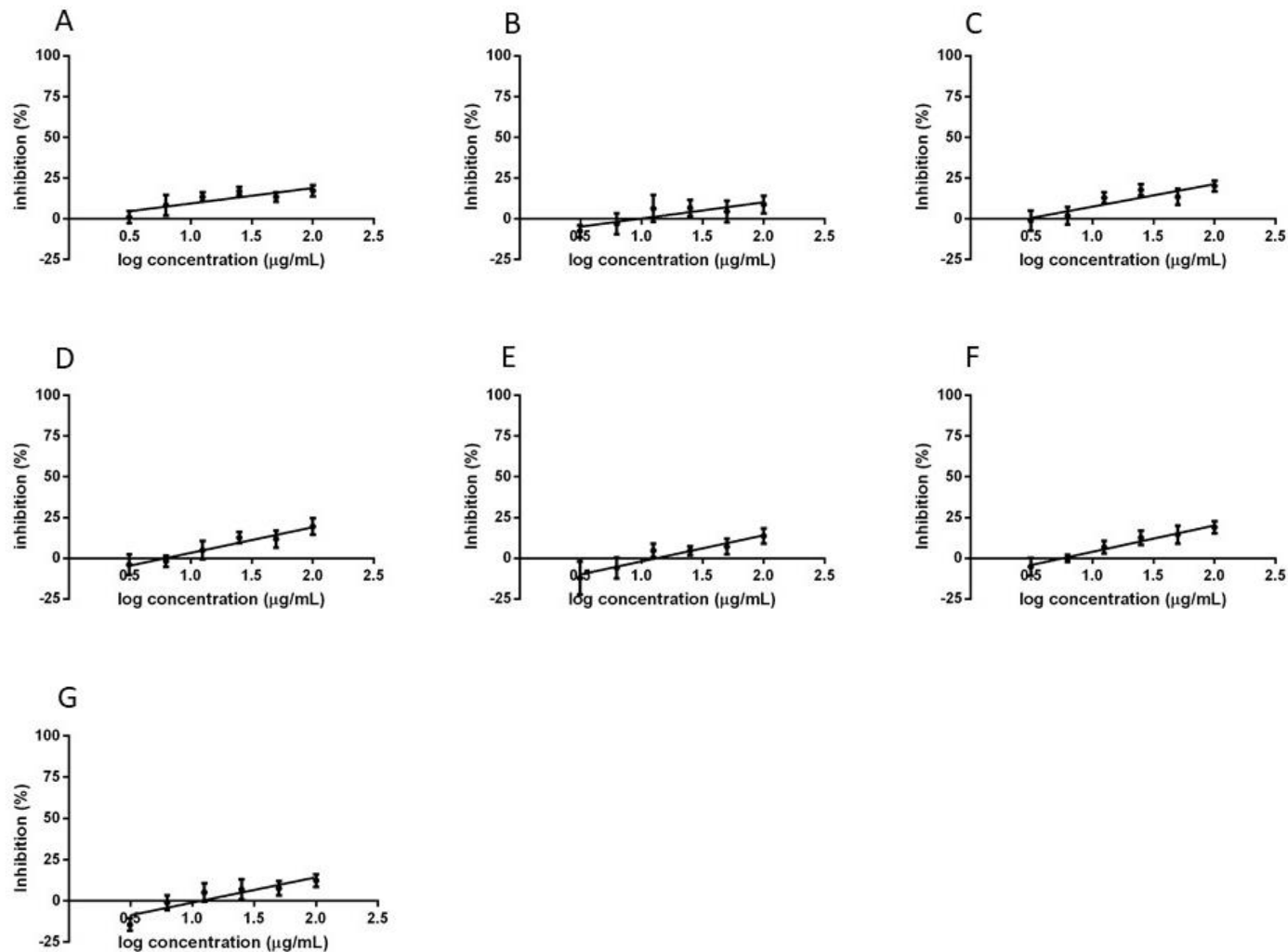


Figure 14: The effect of plant extracts on α -glucosidase activity. A) Water extract of *O. lanceolata*, B) DCM:MeOH extract of *O. lanceolata*, C) chloroform partition extract of *O. lanceolata*, D) water extract of *G. canoargentea*, E) DCM:MeOH extract of *G. canoargentea*, F) chloroform partition of *G. canoargentea* and G) combination plant extract.

A study done on α -glucosidase activity *in vitro* using kaempferol (0.017 mmol/L) and rutin (0.196 mmol/L) compounds reported a high inhibition of 50% of α -glucosidase enzyme activity.¹⁰⁰ In contrast, the present results show low inhibitory activity for α -glucosidase (Figure 13). Although this needs to be confirmed, it is possible that the observed activity is ascribed to the low concentration levels of the compound within the plant extracts. The solvents used in the extraction of *G. canoargentea*, could also be the reason for low activity, as Do *et al.* reported that methanol and ethanol as solvents, extract more active compounds than water extraction in general, thus having a better probability of activity for most studies.¹⁰¹ It is also important to note that the enzyme inhibition discovered from the compounds in the referred studies was achieved using pure, high concentrations, where as in our study only the plant extracts were analyzed. This further qualifies the need to perform compound isolation to further compare the results.

3.3. Cytotoxicity studies

There is a paucity of scientific data regarding the cytotoxicity profiles of the majority of the plant species used as traditional medicines.¹⁰² Scholars such as Patwardhan *et al.* write profoundly on how the use of traditional medicines is predominantly based on anecdotal evidence, and they emphasize the necessity for scientific data that provides information on the toxicity testing thereof.^{103,104} Medicinal plants are potential sources of new drugs; thus, cytotoxicity studies are important in determining the potential toxicity of test samples which may to be developed into pharmaceutical products so as to prevent deleterious effects when used.

The mouse myoblast C2C12 cell line is a sub-clone of murine myoblasts, which in low carbon dioxide levels *in vitro*, differentiate to form plurinucleated syncytia (myotubes).¹⁰⁵ Functionally, myotubes resemble contractile muscle cell which utilize insulin to absorb glucose as a source of energy and thus are an preferred cell line to test for insulin mimetic properties.¹⁰⁵ The Ea.Hy926 cells were used to assess cytotoxicity since the ROS

production that results during T2DM has been scientifically proven to result in progressive endothelial tissue damage.^{106,107}

The organic plant extracts of the individual plant species under study were found not to exhibit a cytotoxicity profile of more than 50% in both the cell lines after 24 h. The *O. lanceolata* DCM:MeOH extract was found to reduce cell density more profoundly ($IC_{50}=100\text{ }\mu\text{g/mL}$) compared to other extracts against Ea. hy926 cell lines (Figure 16B). Exposure of Ea.hy926 cells to *G. canoargentea* chloroform partition and the herbal combination extract resulted in a concentration dependent decrease in cell density. The *G. canoargentea* chloroform partition ($100\text{ }\mu\text{g/mL}$) yielded a 1.6-fold decrease in cell density (Figure 16E) while the plant combination extract had a plateau effect with contestant 1.5-fold decrease inhibition with $3.125\text{--}100\text{ }\mu\text{g/mL}$ extracts (Figure 16G).

A study by Rached *et al.* reported the non-toxic effect of *O. lanceolata* ethyl acetate and n-butanol extracts on PLP2- porcine liver primary cells using 8 mg/mL of extract.¹⁰⁸ These authors did however report that the extracts showed cytotoxicity against MCF-7 (breast cancer) and NCI-H460 (lung carcinoma) cell lines when exposed to $114\text{ }\mu\text{g/mL}$ and $265\text{ }\mu\text{g/mL}$ plant extract. Even though the concentration exhibiting cytotoxicity are not in not comparable with the concentrations used in our study, suggesting the plant species could be selective to cancerous cells.

The DCM:MeOH extract of *O. lanceolata* had a plateaued effect on the C2C12 cells with more than 40% cell density reduction. Analysis of *O. lanceolata* DCM:MeOH extract at low concentrations need to be done so as to confirm if saturation or proliferation is reason of the plateau effect. In C2C12 the DCM:MeOH extract of *G. canoargentea* resulted in a concentration dependent decrease in cell density, with 1.6-fold decrease being observed at $100\mu\text{g/mL}$. The combined herbal extract also exhibited a concentration dependent cell toxicity, with 1.33-fold decrease observed for $100\mu\text{g/mL}$ of extract tested (Figure 15G). Based on the cytotoxicity profile of the combined extract, it can be deduced that the single

water plant extracts treatment, which had >75% cell viability are a safer option than the herbal plant combination of *O. lanceolata* and *G. canoargentea* used by the THP.

Rached and coworkers observed that an aqueous extract of *O. lanceolata* had the highest cell density decrease (50%) when incubated with HepG2 (hepatocellular carcinoma) and Hela (cervical cancer) cell lines at 46 µg/mL and 87 µg/ml respectively.¹⁰⁸

While there remains a paucity of data regarding the toxicity of *G. canoargentea*, methanolic extracts of *Gnidia kraussiana* roots have been reported to be cytotoxic to Hela, HT29 (human colorectal adenocarcinoma) and A431 (squamous carcinoma) cell lines at a concentration of 100µg/mL *in vitro*.¹⁰⁹ The compounds responsible for the cytotoxicity were identified as kaempferol-3-(*p*-coumarpyl)-O-B-D-glucopyranoside, maltol and gnidicin.^{107,108} Kamuhabwa reported 25% cell proliferation inhibition in Hela cells, 50% in HT29 and 75% in A431 cells.¹⁰⁹ These compounds were however not identified in *G. canoargentea* during phytochemical analysis using UPLC-MS in the current study. The absence of these cytotoxic compounds in the *G. canoargentea* extracts in the current study could be attributed to the plant parts used; i.e. previous study investigated the roots, whereas the stem, leaves and flowers were used in the current study.

Kaempferol-3-O-rutinoside isolated from *Morinda morindoides* leaf extract exhibited no cytotoxic effect when tested in MT-4 cells in a study done by Cimanga and partners.¹³³ Marcarini et al, discovered rutin to be not toxic when incubated with HTC hepatic cells over 24 h, however when the cells were incubated with 800 µM of rutin for 72 h, the compound was found to exhibit a cytotoxic profile.¹³⁴ Reports on cytotoxicity studies of 6-hydroxyflavone, pelargonidin-3-O-rutinoside and pelargonidin-3-O-glucoside were not found.

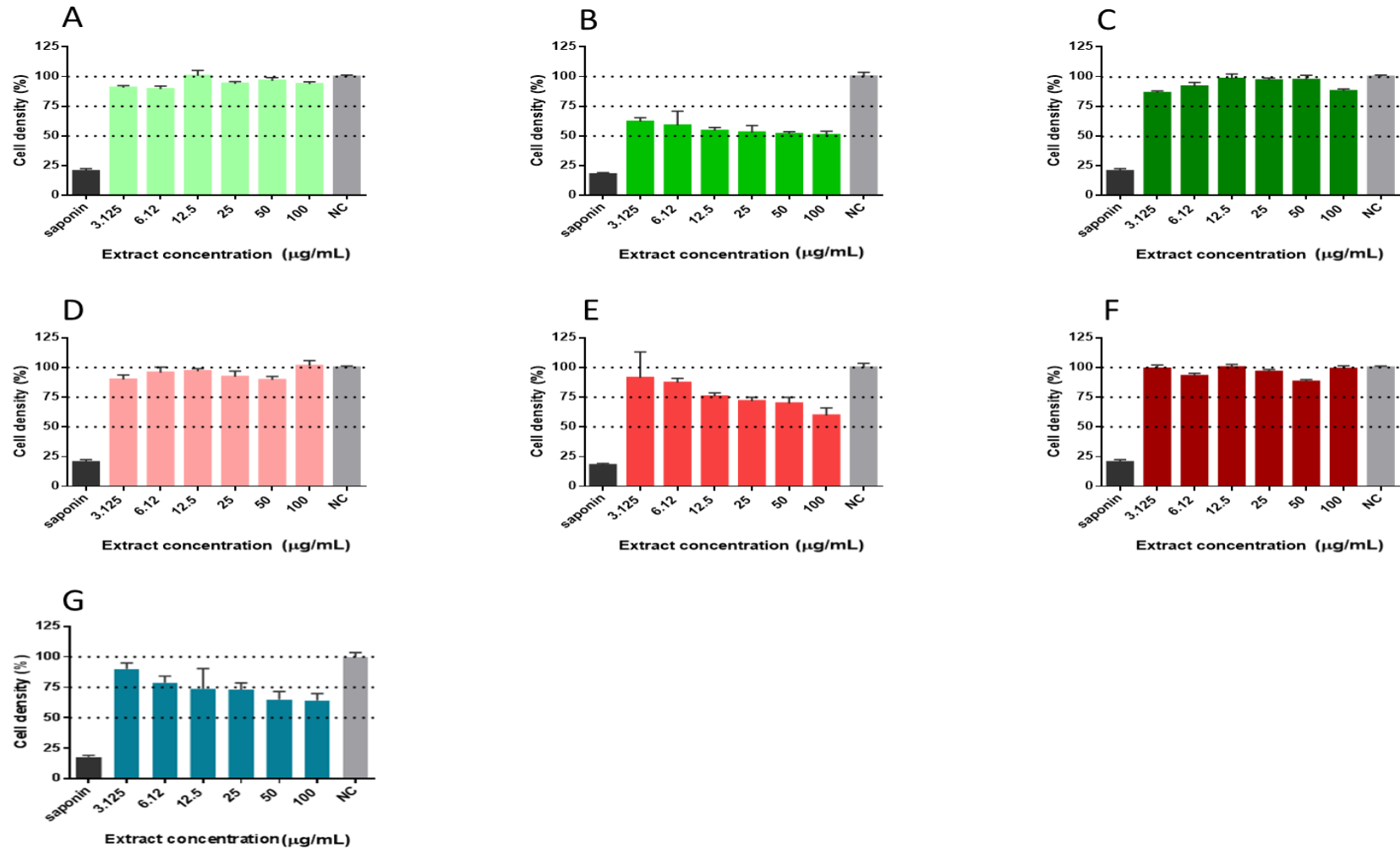


Figure 15: C2C12 cell viability after 24 h exposure to A) water extract of *O. lanceolata*, B) DCM:MeOH extract of *O. lanceolata*, C) chloroform partition extract of *O. lanceolata*, D) water extract of *G. canoargentea*, E) DCM:MeOH extract of *G. canoargentea*, F) chloroform partition of *G. canoargentea* and G) combination plant extract. NC = negative control; saponin = positive control.

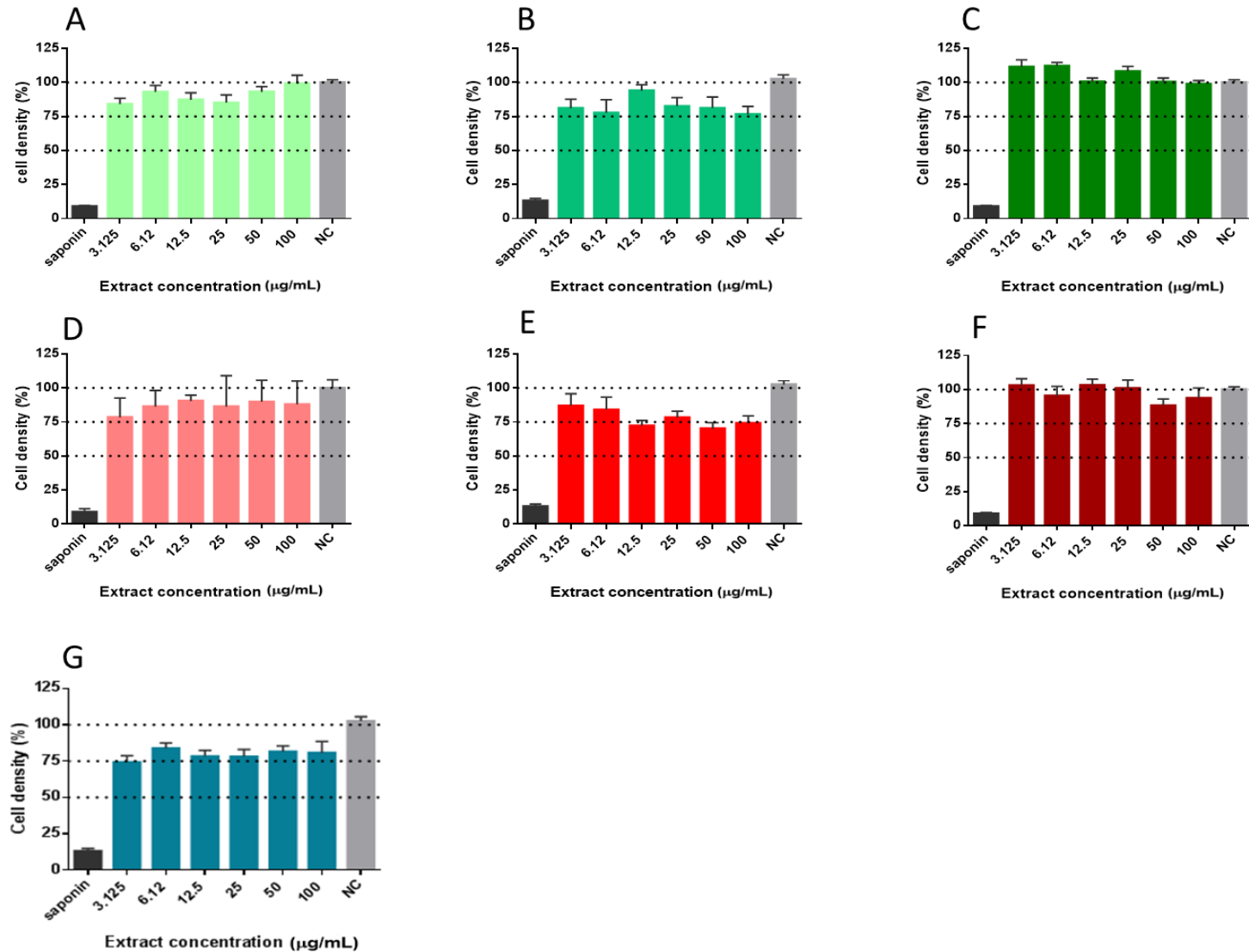


Figure 16: Cell viability of Ea. Hy926 cells after 24 h exposure to A) water extract of *O. lanceolata*, B) DCM:MeOH extract of *O. lanceolata*, C) chloroform partition extract of *O. lanceolata*, D) water extract of *G. canoargentea*, E) DCM:MeOH extract of *G. canoargentea*, F) chloroform partition of *G. canoargentea* and G) combination plant extract NC = negative control; Saponin = positive control.

3.4. Antioxidant activity

T2DM has been associated with increased production of ROS. Both the ABTS and DPPH assay allow for monitoring the ability of samples to quench the formation of free radicals. The radical that is formed in the ABTS assay allows for hydrophilic and hydrophobic antioxidant identification as this radical is not affected by ionic strength.¹¹⁹

The DPPH assay offers a stable nitrogen radical with a purple color that when quenched, turns yellow.¹¹² The DPPH assay is based on the extract and compound's ability to break down the stable radical, 2,2-diphenyl-1-picrylhydrazyl.¹¹² The DPPH radical when reacting with an antioxidant particle, reduces to a corresponding hydrazine.¹¹² This assay is of importance as it has been established to understand how oxidative stress plays a key role in the pathogenesis of DM. The extracts of *G. canoargentea* had more potent radical scavenging ability than that of *O. lanceolata* (Table 6). The DCM:MeOH extract of *G. canoargentea* had low IC₅₀ in both the DPPH and ABTS assay, being considered the best radical scavenger. None of the plant extracts exhibited radical scavenging capabilities that were better than the used positive control, Trolox.

Table 6: *In vitro* antioxidant capacity of *O. lanceolata* and *G. canoargentea* plant extracts.

Extract	Solvent	DPPH (µg/mL)	ABTS (µg/mL)
<i>O. lanceolata</i>	DCM:MeOH	>100	23.51 ± 1.07
<i>O. lanceolata</i>	Water	>100	13.79 ± 1.04
<i>O. lanceolata</i>	Chloroform	61.70 ± 1.03	6.48 ± 1.06
<i>G. canoargentea</i>	DCM:MeOH	18.95 ± 1.03	< 3.125
<i>G. canoargentea</i>	Water	21.86 ± 1.04	3.55 ± 1.02
<i>G. canoargentea</i>	Chloroform	26.40 ± 1.02	< 3.125
Trolox		6.44 ± 1.07	1.66 ± 1.11

The minimal antioxidant activity observed in *O. lanceolata* is surprising as the extract was found to contain the flavonoid, kaempferol. Kaempferol has been shown to have radical scavenging activity (1.14 mM ± 0.02) in an *in vitro* study using the TEAC assay.¹¹⁴ Yeboah

et al found *O. lanceolata* to have antioxidant activity.¹¹⁵ The IC₅₀ obtained by Yeboah and partners for the water and methanol extract was $48.4 \pm 0.4 \mu\text{g/mL}$ and $49.5 \pm 1.9 \mu\text{g/mL}$, respectively, while in this study, the DCM:MeOH partition and water extract samples yielded an IC₅₀ >100 $\mu\text{g/mL}$ (Table 5). *O. lanceolata* has also been found to have more potent activity than Trolox (42 $\mu\text{g/mL}$), with an IC₅₀ of 5 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$ for the ethyl acetate and butanol extract respectively.¹⁰⁸

Rached *et al* ascribed the antioxidant activity observed for *O. lanceolata* to phenolic acid and flavonoid compounds present within the plant.¹⁰⁸ Flavonoids are excellent free radical scavengers, as they are highly reactive electron donors, thus the result observed for *O. lanceolata*, which was shown to contain flavonoids, is unexpected.^{114,117} The flavonoid 6-hydroxyflavone has been found to be present in various plant species (*Urtica dioica*, *Bidens pilosa*, and *Leonotis nepetifolia*) that have been used traditionally to treat diabetes.¹¹⁸

Kaempferol-3-rutinoside, present in *G. canoargentea*, is a flavonol that is a glycosidic derivative of kaempferol.^{118,120} Zielinska and partners found rutin to play a role in protecting cells from oxidative damage,¹²² whereas Fuentes and partners observed improved endothelium-dependent relaxation and vascular contraction in alloxan-induced diabetes studies *in vivo*.¹²⁰ Kamalakkan *et al.* reported that oral administration of rutin (100 mg/kg) to rats with streptozotocin induced diabetes caused a decrease in peroxidation products and upregulated the enzyme and non-enzymatic antioxidant activity *in vivo*.¹²⁴ This implies that plants containing rutin may assist in managing oxidative stress in diabetic patients. Furthermore, these compounds have also been reported to have antioxidant activity, as well as vasorelaxation activity *in vitro*.¹²⁴

The resulting ROS in humans actively attacks the viable pancreatic β -cells which reduces functionality and thus leads to life threatening complications. Intracellularly, the increased production of ROS due to hyperglycaemia results in endothelial damage.

This is the first study to investigate radical scavenging activity of *G. canoargentea*. All the *G. canoargentea* extracts in the ROS study behaved in a similar manner in that they exhibited ROS production inhibition which was less effective than the positive control (Trolox) *in vitro*. *O. lanceolata* extracts exhibited significant ROS inhibition in comparison to the ROS control (cell treated with AAPH only). The *G. canoargentea* (Figure 17 D-F) exhibited an inverse effect, where with increasing plant extract concentration, the ROS in well increased, which would suggest that *O. lanceolata* (Figure 17 A-C) is a better ROS inhibitor than *G. canoargentea*. The observed inverse effect in the *G. canoargentea* extract could be a result of extract exhibiting spontaneous fluorescence, in future studies control regarding this aspect therefore should be considered. However, it is essential to note that both plants were weaker inhibitors of ROS than the positive control, Trolox.

3.5. Glucose uptake

Insulin resistance and secretion deficiency is another hallmark of T2DM and contributes to reduced glucose uptake in cells.¹²⁵ Thus, there is a need for alternative treatments that offer insulin mimetic properties that will decrease the elevated concentration of blood glucose in patients. Glucose uptake assays aim to analyze a plant extract or the isolated compounds ability to enhance glucose uptake in cells. The assay principle involves starving cells, then exposing them to insulin or plant extracts. The insulin binds to insulin receptors resulting in a signaling cascade that translocate GLUT4 ligands to the cell surface; allowing for the uptake of glucose.^{57,125} If the plant extracts have insulin mimetic effects, 2-[N-(7-nitrobenz-2-oxa-1,3-dioxol-4-yl)amino]-2-deoxyglucose (fluorescent analogue of 2-deoxyglucose) will be transported into the cells.

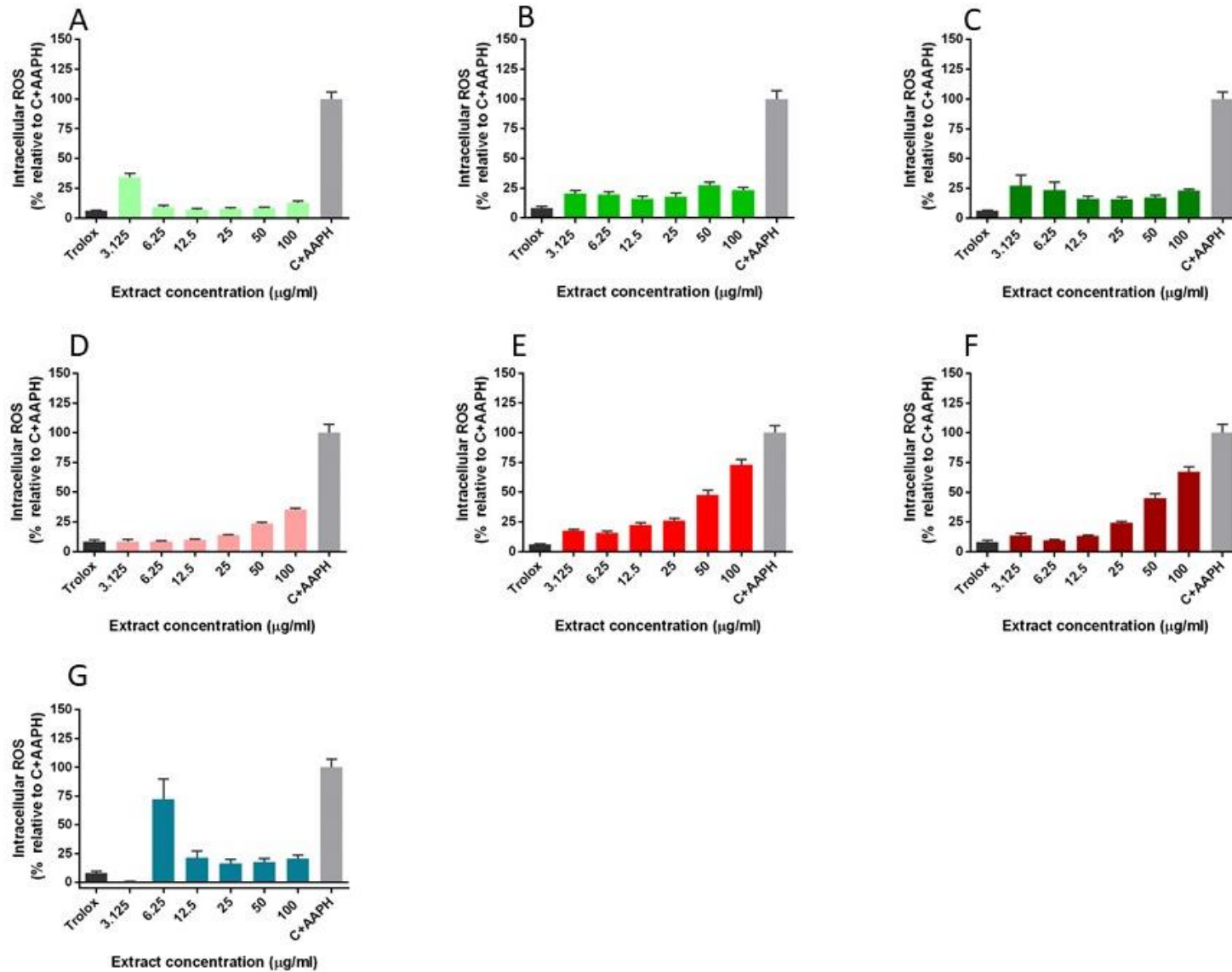


Figure 17: The effect of A) water extract of *O. lanceolata*, B) DCM:MeOH extract of *O. lanceolata*, C) chloroform partition extract of *O. lanceolata*, D) water extract of *G. canoargentea*, E) DCM:MeOH extract of *G. canoargentea*, F) chloroform partition of *G. canoargentea* and G) combination plant extract on intracellular ROS concentrations induced with AAPH. C+AAPH = ROS control; Trolox = positive control.

In Figure 18, C2C12 cells, *O. lanceolata* and *G. canoargentea* did exhibit a level of glucose uptake in C2C12 compared to the negative control (except for the *G. canoargentea* water extract). However, the insulin mimetic effect was insignificant compared to the positive control insulin (Figure 18). The positive control, insulin, was more effective in inducing glucose uptake than any of the plant extracts (Figure 18).

The present study showed fluorescent intensity from the cells incubated with the extract to not be significantly different to the negative control (cells incubated with medium). There was an insignificant increase in 2-NBDG uptake when cells were treated with the organic and chloroform extracts (Figure 18 B, C, E and F), whereas insulin (positive control) was significantly effective in improving glucose uptake. The water extracts of the plants were found not to be effective in promoting glucose uptake, even at the highest concentration tested (Figure 18 A, D and G).

Kaempferol is a phenolic compound that has been shown to exhibit antioxidant and antidiabetic properties where the compound assists in glucose uptake by alleviating the insulin resistance observed in diabetic subjects.¹²⁶⁻¹²⁸ Ezenyi *et al* reported increased intrahepatic glucose uptake in rats that were administered 1000 mg/kg methanol leaf extract of *Syzygium guineense* (which contains kaempferol-3-rutinoside).¹²⁸ The extract prepared from the flower of *Sambucus nigra* has been reported to contain kaempferol-3-rutinoside, and was also found to increase glucose uptake more than insulin in porcine myotubes exposed to 6-600 µg/mL of extract.¹²⁹

Rutin at a concentration of 500 µM was reported to exhibit a strong affinity to assist cells in glucose uptake in an *in vitro* study using rat soleus muscles.¹³² Kappel and partners further discovered that rutin's mechanism of action is similar to that of insulin.¹²⁹ The literature denotes that the commercially available compounds have the ability to promote glucose uptake in *in vitro* studies, being far more effective than the extracts that were analyzed in this study. This implies that the water extraction method that is mostly used

by the THPs may not necessarily result to the extraction of chemical compounds with insulin mimetic effects in glucose uptake.

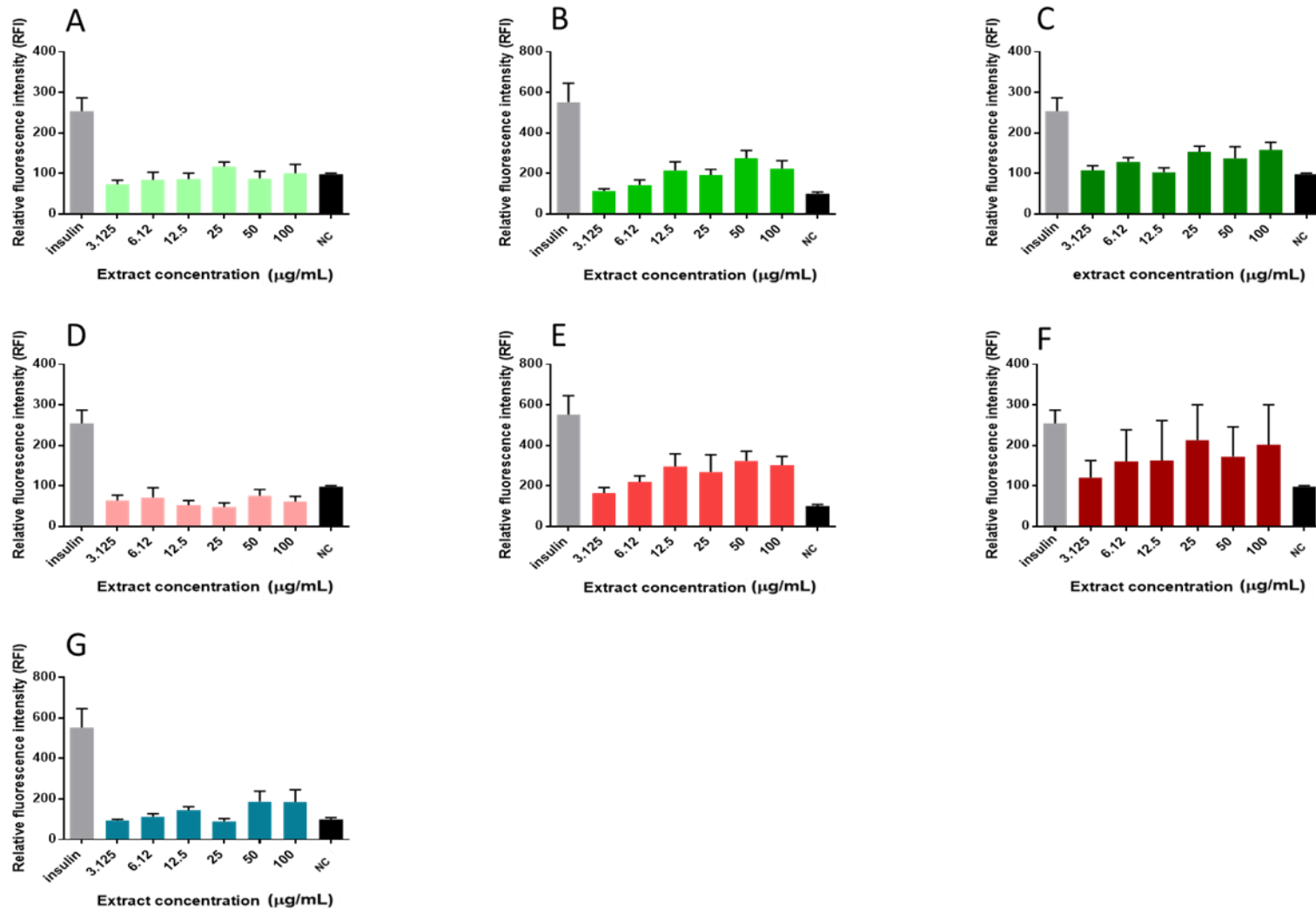


Figure 18: Glucose uptake into C2C12 myotubes after treatment with A) water extract of *O. lanceolata*, B) DCM:MeOH extract of *O. lanceolata*, C) chloroform partition extract of *O. lanceolata*, D) water extract of *G. canoargentea*, E) DCM:MeOH extract of *G. canoargentea*, F) chloroform partition of *G. canoargentea* and G) combination plant extract. Insulin served as a positive control; NC = negative control.

Chapter 4: Conclusion

Plant extracts were prepared ethnomedicinally (hot water extraction) and in a pharmaceutical manner. The purpose of using the pharmaceutical simulant extraction method was to provide a comparative analysis to the extraction obtained following the traditional practitioner's method (hot water extraction). Using the extracts, a phytochemical overview was attained and the phytochemical classes such as phenolics identified from the plant extracts correlated with what was described in literature concerning *O. lanceolata*. Although there were minor differences observed compared to other studies, this may be attributed to the geographical location, season and the method used to obtain extracts. Phytochemicals identified in *G. canoargentea* in the current study have not been identified before within the species and thus is the first study to report on this.

Both *G. canoargentea* and *O. lanceolata* analysed were found to be rich in flavonoids and phenolic compounds. *G. canoargentea* was a better antioxidant candidate in comparison to *O. lanceolata*. More studies looking at lower plant extract concentrations of *G. canoargentea* is necessary to confirm if the plant can offer better radical scavenging activity than the positive control (Trolox).

The SRB assay method was employed to assess the toxicological profile of the plant species using C2C12 and Ea.Hy926 cells as study models. The chloroform partition of *O. lanceolata* had a significant level of cytotoxicity in C2C12 cells, while the *G. canoargentea* chloroform partition and combination plant extract exhibited concentration dependent cytotoxicity. The dose dependent cytotoxicity observed in the combination plant extract suggest that there needs to be caution when using the traditional practitioner's medicine. The observed cytotoxicity for *O. lanceolata* is contradictory to what is reported in literature, this could be attributed to the plant parts used.

Of the extracts prepared, *G. canoargentea* organic (DCM:MeOH) and *O. lanceolata* (chloroform partition) had inhibitory activity against α -amylase while the combinational extract with the water extracts were unsuccessful in regulating the enzyme activity. This can be attributed the method used to extract the compounds and the presence of kaempferol and the anthocyanins. It has been reported in literature that organic solvents yield extracts with concentrated active compounds than water extract, thus corroborating the inhibition activity observed in this study.

It was observed that all of the extract had insignificant activity with regards to glucose uptake in C2C12 cells when compared to insulin, thus concluding that the plants offer minimal insulin mimetic properties. This was in contradiction to what was reported in literature with regards to the compounds found in the extracts. Furthermore, there was minimal ROS inhibition observed within Ea.Hy926 cells exposed to the plant extract while there was significant radical scavenging activity observed in ABTS and DPPH tests. Thus, it is possible that the mode of action the plants use to regulate glucose levels is by stabilization of the reactive oxygen species which in turn would reduce the damage of the vital β cells needed to manage diabetes condition. The minimal ROS inhibition compared to the positive control observed in Ea.Hy926 cells could be due to the fact that the plant extracts used in the lab have not went through the metabolism process, which could cause activation of medicine. However, this requires more research to understand further as the medicine is already working for the traditional practitioner with his patients. Also, the reduction of glucose in blood by inhibition of the carbohydrate hydrolyzing is a possible mechanism with which the plants assist in diabetes management.

The lack of formalized safety assessment mechanisms and regulation in the usage of herbal remedies pose significant risks for human health when used without a thorough assessment of their toxicity, and mode of action with regards to health condition(s) being treated. Given that a concentration dependent cytotoxicity was induced in C2C12 cells, by the combination plant extract, which assimilated the traditional medicine currently used, there remains a need for great caution therein the use of the herbal remedy.

Based on the results, it is evident that *O. lanceolata* and *G. canoargentea* extracts regulate T2DM by scavenging radicals which play part in the disease progression and also inhibit the carbohydrate hydrolyzing enzyme, α -amylase. Furthermore, *O. lanceolata* and *G. canoargentea* offer minimal to no glucose regulation when extracted with water, neither does the combination of the plants offer improved activity and thus the herbal remedies studied do not offer beneficial attributes to glucose regulation when evaluated with the assays employed in this study.

Limitations and recommendations

The combination plant extract studied was based on the prescription of the traditional practitioner of using 60 g (*O. lanceolata*) and 40 g (*G. canoargentea*). The use of a different ration structure for the solo plant extracts than that of the THP limits the level of comparison that can be achieved from the study. Perhaps having different ratios of the herbal combination which is comparable with all extracts rather than a single ratio would offer insight as to how the herbal remedy can best target the health conditions encouraging progression of diabetes.

The organic plant extracts exhibited minimal activities in most assays even though antidiabetic compounds were tentatively identified in UPLC-MS. It is recommended that the extracts are used to isolate the compounds and that they are assessed with NMR to further analyze the structures. Further studies utilizing isolated compounds from the plants may shed more light on the potential of the medicinal properties of *O. lanceolata* and *G. canoargentea*.

In future studies it is recommended that low concentrations are tested of the plant extract wherein a plateau effect or decrease in cell density by low concentration was observed so as to understand the mechanism of action. With regards to the florescent studies such

as ROS inhibition, a control comparing the level of oxidation taking place is needed so as to show the level of induced oxidation compared to normal oxidative stress.

The colour control for the plant extract is warranted as well in order to eliminate possible spontaneous fluorescence effects by the plant extract. It would be recommended to assess isolated compounds from the plants with regards to inhibition of α -glucosidase and α -amylase and the mode of inhibition. Furthermore, analysis of the compounds in different insulin target tissue could present with other possible targets for anti-diabetic activity.

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Appendix I: Ethics approval letter

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 28 August 2018.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.



UNIVERSITEIT VAN PRETORIA
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Faculty of Health Sciences Research Ethics Committee

26/01/2017

Approval Certificate New Application

Ethics Reference No.: 21/2017

Title: An in vitro investigation into the glucose regulating activity of *Gnidia canoargentea* and *Osyris lanceolata*

Dear Bongimpilo Kevin Mkhize

The **New Application** as supported by documents specified in your cover letter dated 3/12/2016 for your research received on the 3/01/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 25/01/2017.

Please note the following about your ethics approval:

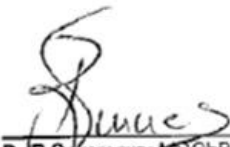
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (**21/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:

- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely



Dr R. Semmer: MBChB; MMed (Int); MPharm, PhD

Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

Appendix II: Reagents and their preparation

3,5-Dinitrosalicylic acid

3,5-Dinitrosalicylic acid powder was purchased from Sigma-Aldrich (St. Louis, USA).

4-Nitrophenyl α -D-glucopyranoside

4-Nitrophenyl α -D-glucopyranoside powder was purchased from Sigma-Aldrich (St. Louis, USA).

2-NBDG

2-NBDG powder was purchased from Invitrogen (USA).

2,2'-Azobis(2-methylpropionamide) dihydrochloride (AAPH)

AAPH powder was purchased from Sigma-Aldrich (St. Louis, USA). A stock solution (400mM) was prepared by dissolving 21.7 mg in 200 μ L of distilled water. Aliquots were stored at -80°C and a fresh working solution was prepared prior to use.

DMSO

DMSO was purchased from Sigma-Aldrich (St Louis, USA) in concentrated form.

Sulforhodamine B

Sulforhodamine B powder was obtained from Sigma-Aldrich (St. Louis, USA) and was prepared by dissolving 57 mg in 100 mL 1% acetic acid.

ABTS^{•+}

ABTS powder was purchased from Sigma-Aldrich (St.Louis, USA).

Acarbose

Acarbose powder was purchased from Sigma-Aldrich (St. Louse, USA).

Dihydrodichlorofluorescein diacetate (H₂-DCF-DA)

Dihydrodichlorofluorescein diacetate powder was obtained from Sigma-Aldrich (St Louis, USA). Stock solutions of 20.5 mM were prepared by dissolving 10 mg of H₂-DCF-DA powder in 1 mL DMSO. Aliquots were stored at -80°C. A fresh working solution was prepared from the aliquots prior to use.

Dulbecco's Modified Eagle's Medium

DMEM was supplied by Sigma-Aldrich (St Louis, USA). A mass of 48 g DMEM powder was dissolved in 5 L sterile deionized water and filter sterilized twice using 0.22 µm cellulose acetate filter. The medium was fortified with 1% penicillin/streptomycin and stored at 4°C. Supplementation of medium with 10% FCS was done prior to use.

Foetal calf serum

Foetal calf serum was purchased from PAA (Pasching, Austria) and was heat inactivated by incubating at 56°C for 45 min prior to use.

Folin Ciocalteu reagent

Folin Ciocalteu reagent was purchased from Sigma-Aldrich (St. Louis, USA) and used in a diluted format.

Insulin

Insulin powder was purchased from Sigma-Aldrich (St. Louis, USA).

Phosphate buffer saline (PBS)

BBL™ FTA hemagglutination powder was obtained from BD bioscience (France). A mass of 9.23 g was dissolved in 1 litre distilled water and stored at 4°C.

Potato starch

Potato starch powder was purchased from Sigma-Aldrich (St. Louis, USA).

Rat intestinal acetone powder

Rat intestinal acetone powder was purchased from Sigma-Aldrich (St. Louis, USA).

Trypan Blue counting solution

Trypan blue powder was obtained from BDH laboratories Supplies (UK). A mass of 200 mg of trypan blue powder was dissolved in 50 ml PBS to obtain 0.1% (w/v) solution which was filtered through a 0.45 μ m syringe filter to remove insoluble particles.

Trolox

Trolox powder was purchased from Sigma-Aldrich (St. Louis, SA).

Solvents

Organic solvents; Dichloromethane ($\geq 99.9\%$ purity), Ethyl acetate ($\geq 99.5\%$ purity), Chloroform ($\geq 90\%$ purity), Methanol ($\geq 99.8\%$ purity), Sulfuric acid ($\geq 97\%$ purity), Toluene ($\geq 99.9\%$ purity) were supplied by Merck chemicals (Darmstadt, Germany).